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(54) Title: LIBRARY SCREENING AS A STRATEGY TO CLONE DRUGS FOR G PROTEIN COUPLED RECEPTORS

(57) Abstract

The present invention is directed to a strategy to identify small peptides that activate any G protein coupled receptor (GPCR) or inactive any constitutively active GPCR by screening combinatorial peptide libraries. The invention comprises expressing a peptide of a peptide library tethered to a GPCR of interest in a cell, and monitoring the cell to determine whether the peptide is an agonist or negative antagonist of the GPCR of interest. The peptide is tethered to the GPCR by replacing the amino terminus of the GPCR with the amino terminus of a self-activating receptor, and replacing the natural peptide ligand present in the amino terminus with the library peptide. In one embodiment for discovery of agonists, a ligand of the self-activating receptor is used to cleave the resulting amino terminus to expose the peptide of the peptide library. In another embodiment for discovery of agonists or negative antagonists, the GPCR construct ends in the peptide so the peptide is always exposed. Preferably, the self-activating receptor is the thrombin receptor and the ligand of the self-activating receptor is thrombin.

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LIBRARY SCREENING AS A STRATEGY TO CLONE DRUGS FOR G PROTEIN COUPLED RECEPTORS

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FIELD OF THE INVENTION

The present invention relates to drug discovery, and more particularly to a strategy to clone drugs for G protein coupled receptors.

BACKGROUND OF THE INVENTION

Throughout this application various publications are referenced, many in parenthesis. Full citations for these publications are provided at the end of the Detailed Description. The disclosures of these publications in their entireties are hereby incorporated by reference in this application.

It has been estimated that more than 50% of the drugs in clinical use today are directed at G protein coupled receptors (GPCRs). Small peptides can activate a number of receptors of this family, such as receptors for thyrotropin-releasing hormone (TRH), which is a tripeptide (Gershengorn and Osman 1996), thrombin, for which a hexapeptide is a full agonist (Tapparelli et al. 1993), and formyl-Met-Leu-Phe, which is a tetrapeptide (Perez et al. 1994). Small molecules can inactivate constitutively active GPCRs, such as benzodiazepines, which inactivate TRH receptor mutants that are constitutively active (Heinflink et al. 1995)(a constitutively active receptor is one that signals in the absence of agonist).

It appears that these small molecules interact primarily, if not exclusively, with the transmembrane (TM) bundle or extracellular (EC) loops of GPCRs (Cascieri et al. 1995). For example, it

appears that the "activation domain" of a GPCR with a large EC amino terminus, such as the receptor for calcitonin, is present within the region of the receptor from the beginning of TM helix one to the C-terminus, which includes the TM bundle and EC loops (Stroop et al. 1995).

The discovery of peptides that could activate GPCRs or inactivate constitutively active GPCRs may have enormous potential for clinical applications because a number of peptide agonists of GPCRs are currently used therapeutically and diagnostically. In the shorter term, the discovery of such peptides will yield reagents that could be used by pharmaceutical companies to identify ligands for or functions of "orphan" receptors.

SUMMARY OF THE INVENTION

To this end, it is an object of the subject invention to provide a strategy to discover small peptides that will activate any G protein-coupled receptor (GPCR) or inactivate any constitutively active GPCR. These peptides could serve as lead chemicals for design of clinically useful drugs or could be used to identify the natural ligand or physiologic function of "orphan" receptors, that is, putative receptors that have been identified (i.e., cloned) but for which the function is unknown. The strategy uses combinatorial peptide libraries tethered to the GPCR. With this approach, millions of random peptides of a given length can be tested for activity in the context of a library and those that activate GPCRs or inactivate constitutively active GPCRs can be identified.

The invention thus provides a method of identifying peptide agonists or negative antagonists of a G protein coupled receptor of interest. The method comprises expressing a peptide of a peptide library tethered to a G protein coupled receptor of interest in

a cell, and monitoring the cell to determine whether the peptide is an agonist or negative antagonist of the G protein coupled receptor of interest.

In one embodiment for identifying peptide agonists, the expression of a peptide of a peptide library tethered to a G protein coupled receptor of interest in a cell comprises preparing a G protein coupled receptor construct, introducing the G protein coupled receptor construct into a cell, allowing the cell to express the G protein coupled receptor encoded thereby, and exposing the cell to a ligand of a self-activating receptor, wherein the ligand cleaves the G protein coupled receptor construct so as to expose the inserted peptide of the peptide library. The G protein coupled receptor construct for identifying a peptide agonist, which is also provided by the subject invention, comprises a nucleic acid molecule encoding a G protein coupled receptor with a deleted first amino terminus; a nucleic acid molecule encoding a second amino terminus of a self-activating receptor attached to the nucleic acid molecule encoding the G protein coupled receptor at the deleted first amino terminus, the second amino terminus having a deleted portion which is a peptide agonist for activating the self-activating receptor; and a nucleic acid molecule encoding the peptide of the peptide library inserted into the second amino terminus and replacing the deleted portion.

In a further embodiment for identifying peptide negative antagonists, the G protein coupled receptor of interest is a constitutively active G protein coupled receptor and the expression of a peptide of a peptide library tethered to the G protein coupled receptor of interest in a cell comprises preparing a constitutively active G protein coupled receptor construct, introducing the constitutively active G protein coupled receptor construct into a

cell, and allowing the cell to express the constitutively active G protein coupled receptor encoded thereby. The constitutively active G protein coupled receptor construct for identifying a peptide negative antagonist, which is also provided by the subject invention, comprises a nucleic acid molecule encoding a constitutively active G protein coupled receptor with a deleted first amino terminus; a nucleic acid molecule encoding a second amino terminus of a self-activating receptor attached to the nucleic acid molecule encoding the constitutively active G protein coupled receptor at the deleted first amino terminus, the second amino terminus having a deleted portion which includes a peptide agonist for activating the self-activating receptor as well as any amino acids positioned amino terminally to the peptide agonist; and a nucleic acid molecule encoding the peptide of the peptide library inserted into the second amino terminus and replacing the deleted portion.

In a still further embodiment for identifying peptide agonists, the expression of a peptide of a peptide library tethered to a G protein coupled receptor of interest in a cell comprises preparing a G protein coupled receptor construct, introducing the G protein coupled receptor construct into a cell, and allowing the cell the express the G protein coupled receptor encoded thereby. The G protein coupled receptor construct for identifying a peptide agonist, which is also provided by the subject invention, comprises a nucleic acid molecule encoding a G protein coupled receptor with a deleted first amino terminus; a nucleic acid molecule encoding a second amino terminus of a self-activating receptor attached to the nucleic acid molecule encoding the G protein coupled receptor at the deleted first amino terminus, the second amino terminus having a deleted portion which includes a peptide agonist for activating the self-activating

receptor as well as any amino acids positioned amino terminally to the peptide agonist; and a nucleic acid molecule encoding the peptide of the peptide library inserted into the second amino terminus and replacing the deleted portion.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features and advantages of this invention will be evident from the following detailed description of preferred embodiments when read in conjunction with the accompanying drawings in which:

Fig. 1 is a diagram of a G protein coupled receptor;

Fig. 2 is a diagram of a thrombin receptor;

Fig. 3 is a diagram of a peptide of a peptide library;

Fig. 4 is a diagram of a G protein coupled receptor construct according to the subject invention;

Fig. 5 is a diagram of a constitutively active G protein coupled receptor construct according to the subject invention;

Fig. 6 is a diagram of the putative two-dimensional topology of the human calcitonin receptor;

Fig. 7 is a diagram of the putative two-dimensional topology of the human herpesvirus-8 GPCR;

Fig. 8 is a diagram of the putative two-dimensional topology of the chimera ThrR/HHV8 GPCR as it is predicted to be in the cell surface membrane of transfected COS-1 cells; and

Fig. 9 is a plasmid map of pcDNA3PROLACFLAGhTHRR/hFSHR.

DETAILED DESCRIPTION

The invention provides a strategy that is designed to discover small peptides that will activate any G protein-coupled receptor (GPCR) or inactivate any constitutively active GPCR. A constitutively active

receptor is one that signals in the absence of an agonist. These peptides could serve as lead chemicals for design of clinically useful drugs or could be used to identify the natural ligand or physiologic function of "orphan" receptors, that is, putative receptors that have been identified (cloned) but for which the function is unknown. The discovery of peptides that could serve these functions may be accomplished with an approach that uses combinatorial peptide libraries. With this approach, millions of random peptides of a given length are tested for activity in the context of a library and those that activate GPCRs or inactivate constitutively active GPCRs are discovered. As stated above, this approach may have enormous potential for clinical applications because a number of peptide agonists of GPCRs are currently used therapeutically and diagnostically. In the shorter term, however, this technology will yield reagents that could be used by pharmaceutical companies to identify ligands for or functions of "orphan" receptors.

To discover small peptides that can serve as agonists for GPCRs, a combinatorial peptide library is constructed that expresses random pentapeptides tethered to the seven TM helical bundle of any GPCR. A pentapeptide library was chosen based on the fact that TRH is a tripeptide that is blocked at both ends (3+2 (for block) =5) and the resulting number of clones is workable.

The library contains all 20 natural amino acids at each of the five positions and therefore has a complexity of $20^5 = 3.2 \times 10^6$ possible combinations. To this end the complementary DNA (cDNA) sequence that normally encodes any GPCR's N-terminal EC domain is substituted by a DNA sequence that encodes the N-terminal ectodomain of a self-activating receptor such as the thrombin receptor. Thrombin receptor (ThrR) is a GPCR that is activated by a mechanism that is

different from most GPCRs. Thrombin is a serine protease that binds to and cleaves its receptor's N-terminal end at a specific site, exposing a new N-terminus that acts as a peptide agonist tethered to the remainder of the receptor molecule. The chimeric ThrR/GPCR has the variable pentapeptide sequence substituting for the native peptide sequence that is normally unmasked by thrombin action and constitutes the ThrR peptide agonist, but retains thrombin binding sequences and the thrombin-specific cleavage site. Therefore, the N-terminus of expressed receptors is cleaved by thrombin at the appropriate location exposing a new N-terminus that is made of the variable pentapeptide segment of the library tethered to the remainder of the GPCR. As used herein, a receptor that operates in this manner is referred to as a self-activating receptor since a ligand of the receptor cleaves the receptor to expose a natural peptide agonist which activates the receptor. Thrombin is the most well known of such self-activating receptors, but the invention can be readily practiced using other such receptors (e.g., the protease activated receptor or a synthetic receptor).

The cDNA sequence encoding the new N-terminus of the chimeric ThrR/GPCR, consisting of a prolactin leader or signal peptide, followed by the FLAG epitope, followed by the N-terminus of the mature human ThrR, where the pentapeptide library is constructed, is constructed by gene synthesis. The cDNA sequence consists of a DNA segment of approximately 300 base pairs encoding 100 amino acids that is ligated in frame through an appropriate restriction endonuclease cleavage site created by polymerase chain reaction (PCR) in the cDNA of any GPCR at a position encoding the amino acids that constitute the transition between the N-terminus and the first TM domain. After ligation into a mammalian expression vector, *Escherichia coli* is

transformed by electroporation and the transformants are subdivided into pools whose maximal workable complexity is determined according to the efficiency of mammalian cell transfection and/or sensitivity of the detection system.

Amplified reporter systems based on the second messenger systems triggered by the GPCR are used. For discovery of agonists, the assay is based on gene induction in COS-1 cells using β-galactosidase as a reporter gene in a single cell assay. This assay takes advantage of the amplification of the enzyme activity of the reporter, with an easily determined color reaction as endpoint, and of the expression of a single receptor clone with its tethered agonist in COS-1 cells because of replication of the plasmids introduced. The signal is increased because the construct used has a nuclear localization signal ligated to the β-galactosidase that allows the protein to concentrate in the nucleus (Hersh et al. 1995). Single clones that exhibit activation of chimeric ThrR/GPCR after thrombin addition to cleave the N-terminus and expose the tethered agonist, as measured by increased color reaction, are isolated using sib selection, which consists of successive subdivision and amplification of positive pools of clones. A number of other reporter systems can also be used. These include, but are not limited to, analysis of acute effects of agonist using *Xenopus laevis* oocytes in which one measures changes in membrane conductance - using calcium-activated chloride conductance for phosphoinositide (PI) cascade or cAMP-activated chloride conductance through cystic fibrosis transmembrane regulator (CFTR) that is co-expressed for cAMP cascade; induction of genes in COS-1 cells that yield protein products that are displayed in the cytoplasm or on the surfaces of cells and visualized by immunofluorescence (by microscopy or fluorescence

activated cell sorting) or immunocytochemistry; and analysis of acute effects on elevation of cytoplasmic calcium using fluorescence indicators.

To discover small peptides that can serve as agonists or small peptides that can serve as negative antagonists (or inverse agonists) for GPCRs, a second type of combinatorial peptide library is constructed that expresses random pentapeptides tethered to the seven TM helical bundle of a given GPCR that is different from the one described above to discover agonists but is based on the same principles. This library also contains all 20 natural amino acids at each of the five positions and therefore has a complexity of $20^5 = 3.2 \times 10^6$ possible combinations. In this library, however, the cDNA sequence that normally encodes GPCR's N-terminal EC domain is substituted by a DNA sequence that encodes the self-activating receptor's (e.g., thrombin's) N-terminal ectodomain but without the domain that usually is cleaved to reveal the tethered peptide. In this library, the chimeric ThrR/GPCR has the variable pentapeptide sequence substituting for the native peptide sequence that is normally unmasked by thrombin action exposed as the N-terminus of all receptors. Therefore, the N-terminus of expressed receptors is a random pentapeptide that can act as an agonist of a GPCR or as a negative antagonist with regard to the constitutive activity of some GPCRs. With regard to the negative antagonists, in contrast to looking for stimulation of a GPCR signalling response, monitoring is for inactivation of a "basal" activity.

A two-reporter system is used for discovery of negative antagonists. The second reporter gene is used to identify cells that have been transfected and are expressing foreign proteins and to distinguish them from cells that have not been transfected and are not expressing foreign proteins. This is a crucial

distinction for this approach because differentiation between cells that have the capacity to express the specific reporter gene but are not because transcription has been inhibited and cells that are not expressing the reporter gene because they are not transfected is necessary. The same reporter genes for GPCR-specific effects as for the discovery of agonist peptides are used. The nonspecific reporter for transfection is a construct containing a mutant of the human placental alkaline phosphatase gene (Tate et al. 1990) that is targeted to the cytoplasm under the control of a cytomegalovirus promoter. Thus, one can monitor for 3 types of cells: 1) cells in which β-galactosidase is expressed at high levels in the nucleus and alkaline phosphatase is expressed in the cytoplasm - these are transfected cells that do not express receptors that contain a peptide that has negative antagonistic activity because expression of β-galactosidase is induced by the constitutive signalling activity of the GPCR; 2) cells in which β-galactosidase is not expressed in the nucleus and alkaline phosphatase is not expressed in the cytoplasm - these are cells that have not been transfected; and 3) cells in which β-galactosidase is not expressed or is expressed at low levels in the nucleus and alkaline phosphatase is expressed in the cytoplasm - these are transfected cells that express receptors that contain a peptide that has negative antagonistic activity. The approach to sib selection is identical to that outlined above.

A yeast (*Saccharomyces cerevisiae*) bioassay system that is responsive to activation of GPCRs or to inactivation of constitutively active GPCRs can also be used to screen the tethered, combinatorial peptide library. This bioassay is based on the finding that mammalian GPCRs expressed in yeast will regulate the endogenous signal transduction cascades (Dohlman et al.

1991), in particular the pathway for regulation of proliferation (King et al. 1990). A sensitive and specific yeast expression system permits powerful genetic selection methods, which use modifications in the endogenous pheromone response pathways (Price et al. 1995; Price et al. 1996), to be developed for use with the screening methods of the subject invention. The pheromone signalling cascade in yeast uses one of two GPCRs {for (STE2) or a mating factor (STE3)} to couple to a heterotrimeric G protein, which is comprised of (GPA1), (STE4) and (STE18) subunits, to activate a protein kinase signalling cascade that leads to cell cycle arrest, which is mediated by FAR1, and activation of pheromone-responsive genes, such as FUS1. SST2 is another important member of this signalling pathway because it serves to desensitize (or "turn off") the pathway. Several members of this pathway can be modified to improve the sensitivity and assay of GPCRs. This system provides markedly greater ease of assay and permits the screening of hundreds of thousands of recombinant GPCR clones simultaneously. Systems can be developed that can be used to screen for agonist and negative antagonist probes/drugs. The major advantage of this type of assay system over those usually employed to screen numerous potential probes/drugs rapidly, which is necessary for the application of the method of the subject invention, is that it relies on a response in a single yeast cell and will identify the responsive cell in a population of millions of cells.

One assay will be a minor modification of the previously published yeast expression system to assay for activation of GPCRs in which FAR1 and SST2 genes were inactivated and a FUS1-HIS3 gene is used for selection of cells expressing activated GPCRs on a medium deficient in histidine (Price et al. 1995). The changes will involve only adapting the system so that

it will allow high efficiency transformation of yeast cells with a library that contains 3.2 million different GPCRs. The second assay will be modified more extensively so that it will measure constitutively activated GPCRs that are inactivated. One approach to this type of assay will involve using yeast cells in which the FAR1 gene is intact so that constitutively active GPCRs will cause cells to be arrested in the cell cycle. Cells in which the GPCR has been inactivated will not exhibit growth arrest but will proliferate as normal haploid cells in the absence of mating factor.

The invention thus provides a method of identifying peptide agonists or negative antagonists of a G protein coupled receptor of interest. The method comprises expressing a peptide of a peptide library tethered to a G protein coupled receptor of interest in a cell, and monitoring the cell to determine whether the peptide is an agonist or negative antagonist of the G protein coupled receptor of interest.

In one embodiment for identifying peptide agonists, the expression of a peptide of a peptide library tethered to a G protein coupled receptor of interest in a cell comprises preparing a G protein coupled receptor construct, introducing the G protein coupled receptor construct into a cell, allowing the cell to express the G protein coupled receptor encoded thereby, and exposing the cell to a ligand of a self-activating receptor, wherein the ligand cleaves the G protein coupled receptor construct so as to expose the inserted peptide of the peptide library. The G protein coupled receptor construct for identifying a peptide agonist, which is also provided by the subject invention, comprises a nucleic acid molecule encoding a G protein coupled receptor with a deleted first amino terminus; a nucleic acid molecule encoding a second amino terminus of a self-activating receptor attached

to the nucleic acid molecule encoding a G protein coupled receptor at the deleted first amino terminus, the second amino terminus having a deleted portion which is a peptide agonist for activating the self-activating receptor; and a nucleic acid molecule encoding the peptide of the peptide library inserted into the second amino terminus and replacing the deleted portion.

One embodiment of a G protein coupled receptor construct for identifying a peptide agonist of the G protein coupled receptor is shown in Fig. 4. Referring to Figs. 1-4, the construct involves three parts based on a nucleic acid molecule encoding a G protein coupled receptor (10)(Fig. 1), a nucleic acid molecule encoding a thrombin receptor (12)(Fig. 2), and a nucleic acid molecule encoding a peptide (14) of a peptide library (Fig. 3). Referring to Fig. 1, the G protein coupled receptor (10) includes an amino terminus (16). Referring to Fig. 2, the thrombin receptor (12) also includes an amino terminus (18). Within the amino terminus (18) of the thrombin receptor (12) is a portion (20) which is a peptide agonist for the thrombin receptor. When the thrombin receptor is exposed to thrombin, thrombin cleaves the amino terminal part of the molecule (22) leaving the portion (20) which is a peptide agonist exposed. The portion (20) reacts with the remainder of the thrombin molecule and binds thereto, activating the thrombin receptor. Referring to Fig. 3, the peptide (14) of a peptide library is shown.

Fig. 4 shows one embodiment of the G protein coupled receptor construct for identifying a peptide agonist according to the subject invention positioned within a cellular membrane (24). The construct includes a nucleic acid molecule encoding the G protein coupled receptor (10) but a portion of the nucleic acid molecule which encodes the amino terminus of the

receptor is deleted. In its place, the amino terminus (18) of the thrombin receptor is inserted. Within the amino terminus (18) of the thrombin receptor, the portion which is a peptide agonist has been deleted and replaced with the peptide (14) of the peptide library. Thus, the G protein coupled receptor construct has the backbone of a selected G protein coupled receptor, with an amino terminus of the thrombin receptor. However, the normal peptide agonist of the thrombin receptor has been replaced by a peptide library. Thus, when the G protein coupled receptor construct of the subject invention is exposed to thrombin, thrombin will cleave the amino terminal part (22) of the construct leaving the peptide (14) of the peptide library exposed. If the exposed peptide is an agonist of the G protein coupled receptor, the receptor will be turned on.

In a further embodiment for identifying a peptide negative antagonist, the G protein coupled receptor of interest is a constitutively active G protein coupled receptor and the expression of a peptide of a peptide library tethered to the G protein coupled receptor of interest in a cell comprises preparing a constitutively active G protein coupled receptor construct, introducing the constitutively active G protein coupled receptor construct into a cell, and allowing the cell to express the constitutively active G protein coupled receptor encoded thereby. The constitutively active G protein coupled receptor construct for identifying a peptide negative antagonist, which is also provided by the subject invention, comprises a nucleic acid molecule encoding a constitutively active G protein coupled receptor with a deleted first amino terminus; a nucleic acid molecule encoding a second amino terminus of a self-activating receptor attached to the nucleic acid molecule encoding the constitutively active G protein coupled receptor at the deleted first amino terminus,

the second amino terminus having a deleted portion which includes a peptide agonist for activating the self-activating receptor as well as any amino acids positioned amino terminally to the peptide agonist; and a nucleic acid molecule encoding the peptide of the peptide library inserted into the second amino terminus and replacing the deleted portion.

The constitutively active G protein coupled receptor construct for identifying a peptide negative antagonist of the constitutively active G protein coupled receptor is shown in Fig. 5, positioned within a cellular membrane (24). The construct includes a nucleic acid molecule encoding the G protein coupled receptor (10) but a portion of the nucleic acid molecule which encodes the amino terminus of the receptor is deleted. In its place, the amino terminus (18) of the thrombin receptor is inserted. Within the amino terminus (18) of the thrombin receptor, the portion which is a peptide agonist has been deleted as well as any amino acids positioned amino terminally to the peptide agonist which are normally cleaved by thrombin, and replaced with the peptide (14) of the peptide library. Thus, the constitutively active G protein coupled receptor construct has the backbone of a selected G protein coupled receptor, with an amino terminus of the thrombin receptor. However, the normal peptide agonist of the thrombin receptor has been replaced by a peptide library and the peptide is always exposed. If the exposed peptide is a negative antagonist of the constitutively active G protein coupled receptor, the receptor will be turned off by the exposed peptide.

In a still further embodiment for identifying peptide agonists, the expression of a peptide of a peptide library tethered to a G protein coupled receptor of interest in a cell comprises preparing a G protein coupled receptor construct, introducing the G

protein coupled receptor construct into a cell, and allowing the cell to express the G protein coupled receptor encoded thereby. The G protein coupled receptor construct for identifying a peptide agonist, which is also provided by the subject invention, comprises a nucleic acid molecule encoding a G protein coupled receptor with a deleted first amino terminus; a nucleic acid molecule encoding a second amino terminus of a self-activating receptor attached to the nucleic acid molecule encoding the G protein coupled receptor at the deleted first amino terminus, the second amino terminus having a deleted portion which includes a peptide agonist for activating the self-activating receptor as well as any amino acids positioned amino terminally to the peptide agonist; and a nucleic acid molecule encoding the peptide of the peptide library inserted into the second amino terminus and replacing the deleted portion. This G protein coupled receptor construct for identifying a peptide agonist of a G protein coupled receptor has the same structure as the construct shown in Fig. 5 except that the G protein coupled receptor (10) is not a constitutively active receptor.

The Examples which follow relate to particular GPCRs, such as the human calcitonin receptor, the human follicle-stimulating hormone receptor, and a GPCR of human herpesvirus-8. However, as should be readily apparent to those of ordinary skill in the art, this invention is equally applicable to any GPCR. GPCRs are the largest family of cell surface receptors and act indirectly to regulate the activity of a separate plasma membrane-bound target protein, which can be an enzyme or an ion channel. The interaction between the receptor and the target protein is mediated by a third protein, called a trimeric GTP-binding regulatory protein (G protein). The activation of the target protein either alters the conformation of

one or more intracellular mediators (if the target protein is an enzyme) or alters the ion permeability of the plasma membrane (if the target protein is an ion channel).

GPCRs include, for example, the alpha-adrenergic receptors, the beta-adrenergic receptors, dopaminergic receptors, serotonergic receptors, muscarinic cholinergic receptors, peptidergic receptors, and the thyrotropin releasing hormone receptor. GPCRs are characterized by a seven transmembrane-spanning topology (see Figs. 1, 2, 4-8). As used herein, the amino terminus of a GPCR refers to that portion of the GPCR which is extracellular, extending from the amino end of the GPCR to the first transmembrane domain (the amino terminus is depicted in Figs. 4 and 5).

The various G protein coupled receptor constructs of the subject invention include the amino terminus of a self-activating receptor as defined herein. In one embodiment, the self-activating receptor is the thrombin receptor. The amino acid sequence of this amino terminus of the thrombin receptor is shown in SEQ ID NO:1, with amino acid residues 9 to 13 of SEQ ID NO:1 representing the natural peptide agonist of the thrombin receptor. These residues (9 to 13 of SEQ ID NO:1) are replaced with the peptide library in accordance with the subject invention. In one embodiment of the G protein coupled receptor construct, the amino acids normally cleaved by thrombin (residues 1 to 8 of SEQ ID NO:1) are also replaced by the peptide of the peptide library.

SEQ ID NO:1:
LDATLDPRSFLLRNPNDKYEPFWEDEEKNESGLTEYRLVSINKSSPLQK
QLPAFISEDASGYL

In one embodiment discussed in the Examples, the G protein coupled receptor construct is of a human

calcitonin receptor (see Fig. 6). The human calcitonin receptor construct according to the subject invention has an amino acid sequence as shown in SEQ ID NO:44, wherein amino acid residues 47 to 51 of SEQ ID NO:44 are the peptide of a peptide library, amino acid residues 1 to 101 of SEQ ID NO:44 are the second amino terminus, and amino acid residues 102 to 429 of SEQ ID NO:44 are the nucleic acid molecule encoding the human calcitonin receptor with the first amino terminus deleted.

SEQ ID NO:44:

MDSKGSSQKGSRLLLLLVVSNLLLCQGVVSDYKDDDDKLDATLDPRXXXXXNPNDKYEPF
WEDEEKNESGLTEYRLVSINKSSPLQKQLPAFISEDASGYLVLYYLAIVGHSLSIFTLVI
SLGIFVFFRSLGCQRVTLHKNMFLTYILNSMIIIIHLVEVVPNGELVRRDPVSCKILHFF
HQYMMACNYFWMLCEGIYLHTLIVVAVFTEKQRLRWYYLLGWGFPLVPTTIHAITRAVYF
NDNCWLSVETHLLYIIHGPVMAALVVNFFFLLNIVRVLVTKMRETHEAESHMYLKAVKAT
MILVPLLGIQFVVFPWRPSNKMLGKIYDYVMHSLIHFQGFFVATIYCFCNNEVQTTVKRQ
WAQFKIQWNQRWGRRPSNRSARAAAAAAEAGDIPIYICHQELRNEPANNQGEESAEIIPL
NIIEQESSA

In a further embodiment discussed in the Examples, the G protein coupled receptor construct is of a human follicle stimulating hormone receptor. The human follicle stimulating hormone receptor construct has an amino acid sequence as shown in SEQ ID NO:2, wherein amino acid residues 47 to 51 of SEQ ID NO:2 are the peptide of a peptide library, amino acid residues 39 to 101 of SEQ ID NO:2 are the second amino terminus, and amino acid residues 102 to 436 of SEQ ID NO:2 are the nucleic acid molecule encoding the human follicle stimulating hormone receptor with the first amino terminus deleted.

SEQ ID NO:2:

MDSKGSSQKGSRLLLLLVVSNLLLCQGVVSDYKDDDDKLDATLDPRXXXXXNPNDKYEPFWEDEBK
NESGLTEYRLVSINKSSPLQKQLPAFISEDASGYLGYNILRVLIWFISILAITGNIIVLVILTTSQ
YKLTVPRFLMCNLAFADLCIGIYLLLIASVDIHTKSQYHNYAIDWQTGAGCDAAGFFTVFASELSV
YTLTAITLERWHTITHAMQLDCKVQLRHAASVMVMGWIFAFAAALFPIFGISSYMKVSICLPMDID
SPLSQLYVMSLLVLNVLAFVVICGCYIHIYLTVRNPNIVCSSSDTRIAKRMAMLIFTDFLCMAPIS
FFAISASLKVPLITVSKAKILLVLFHPINSCANPFLYAIFTKNFRRDFFILLSKCGCYEMQAQIYR
TETSSTVHNTHPRNGHCSSAPRVTNGSTYILVPLSHLAQN

As used herein, the term "as shown in" when used in conjunction with a SEQ ID NO for a nucleotide sequence refer to a nucleotide sequence which is substantially the same nucleotide sequence, or derivatives thereof (such as deletion and hybrid variants thereof, splice variants thereof, etc.). Nucleotide additions, deletions, and/or substitutions, such as those which do not affect the translation of the DNA molecule, are within the scope of a nucleotide sequence as shown in a particular nucleotide sequence (i.e. the amino acid sequence encoded thereby remains the same). Such additions, deletions, and/or substitutions can be, for example, the result of point mutations made according to methods known to those skilled in the art. It is also possible to substitute a nucleotide which alters the amino acid sequence encoded thereby, where the amino acid substituted is a conservative substitution or where amino acid homology is conserved. It is also possible to have minor nucleotide additions, deletions, and/or substitutions which do not alter the function of the resulting GPCR. These are also within the scope of a nucleotide sequence as shown a particular nucleotide sequence.

Similarly, the term "as shown in" when used in conjunction with a SEQ ID NO for an amino acid sequence refers to an amino acid sequence which is substantially the same amino acid sequence or derivatives thereof. Amino acid additions, deletions, and/or substitutions which do not negate the ability of the resulting protein (or peptide) to form a functional

protein (or peptide) are within the scope of an amino acid sequence as shown in a particular amino acid sequence. Such additions, deletions, and/or substitutions can be, for example, the result of point mutations in the DNA encoding the amino acid sequence, such point mutations made according to methods known to those skilled in the art. Substitutions may be conservative substitutions of amino acids. Two amino acid residues are conservative substitutions of one another, for example, where the two residues are of the same type. In this regard, alanine, valine, leucine, isoleucine, glycine, cysteine, phenylalanine, tryptophan, methionine, and proline, all of which are nonpolar residues, are of the same type. Serine, threonine, tyrosine, asparagine, and glutamine, all of which are uncharged polar residues, are of the same type. Another type of residue is the positively charged (basic) polar amino acid residue, which includes histidine, lysine, and arginine. Aspartic acid and glutamic acid, both of which are negatively charged (acidic) polar amino acid residues, form yet another type of residue. Further descriptions of the concept of conservative substitutions are given by French and Robson 1983, Taylor 1986, and Bordo and Argos 1991.

As further used herein, the term "as shown in" when used in conjunction with a SEQ ID NO for a nucleotide or amino acid sequence is intended to cover linear or cyclic versions of the recited sequence (cyclic referring to entirely cyclic versions or versions in which only a portion of the molecule is cyclic, including, for example, a single amino acid cyclic upon itself), and is intended to cover derivative or modified nucleotide or amino acids within the recited sequence. For example, those skilled in the art will readily understand that an adenine nucleotide could be replaced with a methyladenine, or a

cytosine nucleotide could be replaced with a methylcytosine, if a methyl side chain is desirable. Nucleotide sequences having a given SEQ ID NO are intended to encompass nucleotide sequences containing these and like derivative or modified nucleotides, as well as cyclic variations. As a further example, those skilled in the art will readily understand that an asparagine residue could be replaced with an ethylasparagine if an ethyl side chain is desired, a lysine residue could be replaced with a hydroxylysine if an OH side chain is desired, or a valine residue could be replaced with a methylvaline if a methyl side chain is desired. Amino acid sequences having a given SEQ ID NO are intended to encompass amino acid sequences containing these and like derivative or modified amino acids, as well as cyclic variations. Cyclic, as used herein, also refers to cyclic versions of the derivative or modified nucleotides and amino acids.

As further used herein, a nucleic acid molecule can be deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), the latter including messenger RNA (mRNA). The nucleic acid can be genomic or recombinant, biologically isolated or synthetic.

The DNA molecule can be a cDNA molecule, which is a DNA copy of an mRNA encoding the protein.

The G protein coupled receptor construct of the subject invention can be expressed in suitable host cells using conventional techniques. Any suitable host and/or vector system can be used to express the GPCR construct. For in vitro expression, bacterial hosts (for example, *Escherichia coli*) and mammalian hosts (for example, COS cells) are preferred. For screening using the GPCR construct in which the inserted peptide is always exposed, yeast cells are preferred. The use of yeast cells as a host for expression of the GPCR construct allows for the

screening for negative antagonists of constitutively active GPCRs or for the screening of agonists of GPCRs. Expression of the construct is desirable to identify peptide agonists and negative antagonists of the GPCR, which can then be used for study and/or research purposes, as well as for therapy of inherited or acquired human disorders related to GPCR function.

Techniques for introducing the construct into the host cells may involve the use of expression vectors which comprise the nucleic acid molecule encoding the construct. These expression vectors (such as plasmids and viruses) can then be used to introduce the nucleic acid molecule into suitable host cells. For example, DNA encoding the construct can be injected into the nucleus of a host cell or transformed into the host cell using a suitable vector, or mRNA encoding the construct can be injected directly into the host cell, in order to obtain expression of the GPCR construct in the host cell.

Various methods are known in the art for introducing nucleic acid molecules into host cells. One method is microinjection, in which DNA is injected directly into the nucleus of cells through fine glass needles (or RNA is injected directly into the cytoplasm of cells). Alternatively, DNA can be incubated with an inert carbohydrate polymer (e.g. dextran) to which a positively charged chemical group (e.g. diethylaminoethyl ("DEAE")) has been coupled. The DNA sticks to the DEAE-dextran via its negatively charged phosphate groups. These large DNA-containing particles, in turn, stick to the surfaces of cells, which are thought to take them in by a process known as endocytosis. Some of the DNA evades destruction in the cytoplasm of the cell and escapes to the nucleus, where it can be transcribed into RNA like any other gene in the cell. In another method, cells efficiently take in DNA in the form of a precipitate with calcium

phosphate. In electroporation, cells are placed in a solution containing DNA and subjected to a brief electrical pulse that causes holes to open transiently in their membranes. DNA enters through the holes directly into the cytoplasm, bypassing the endocytotic vesicles through which they pass in the DEAE-dextran and calcium phosphate procedures (passage through these vesicles may sometimes destroy or damage DNA). DNA can also be incorporated into artificial lipid vesicles, liposomes, which fuse with the cell membrane, delivering their contents directly into the cytoplasm. In an even more direct approach, used primarily with plant cells and tissues, DNA is absorbed to the surface of tungsten microprojectiles and fired into cells with a device resembling a shotgun.

Further methods for introducing nucleic acid molecules into cells involve the use of viral vectors. Since viral growth depends on the ability to get the viral genome into cells, viruses have devised clever and efficient methods for doing it. Various viral vectors have been used to transform mammalian cells, such as vaccinia virus, adenovirus, and retrovirus.

As indicated, some of these methods of transforming a cell require the use of an intermediate plasmid vector. U.S. Patent No. 4,237,224 to Cohen and Boyer describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture. The DNA sequences are cloned into the plasmid vector using standard cloning procedures known in the art, as described by Sambrook et al. (1989).

Host cells into which the nucleic acid encoding the construct has been introduced can be used

to produce (i.e. to functionally express) the GPCR construct. The cell can then be monitored to determine whether the peptide tethered to the GPCR is an agonist or negative antagonist (in the case of a constitutively active GPCR) of the GPCR. The method of monitoring can be chosen based on the signalling pathway of the GPCR, or the construct can further include marker or reporter systems as discussed in further detail below. For example, if the G protein coupled receptor signals through an ion channel pathway, the monitoring can comprise detecting levels of the ion within the cell. If the G protein coupled receptor signals through a calcium ion channel pathway, the cell to be used can be a *Xenopus* oocyte and the monitoring can comprise voltage clamp analysis. If the G protein coupled receptor signals through a cyclic adenosine monophosphate pathway, the monitoring can comprise detecting levels of cyclic adenosine monophosphate within the cell.

The invention further provides a cell comprising the G protein coupled receptor construct of the subject invention, as well as an expression vector comprising the construct. A host cell comprising the expression vector is also provided. Such expression vectors include a plasmid and a virus. Preferably, the cell into which the construct or expression vector (comprising the construct) is introduced is a *Xenopus* oocyte, a mammalian cell (such as COS-1 cells; see Gershengorn and Osman 1996), or a yeast cell.

EXAMPLE I

Peptide Agonists of hFSH-R

A combinatorial peptide library was constructed that expresses random pentapeptides tethered to the seven transmembrane helical bundle of the human follicle-stimulating hormone receptor

(hFSH-R). This library encompasses all 20 natural amino acids at each of the five positions, and, therefore, has a complexity of $20^5 = 3.2 \times 10^6$ possible combinations. To this end, the complementary DNA sequence that normally encodes the hFSH-R's amino terminal extracellular domain was substituted by a DNA sequence that encodes the thrombin receptor's amino terminal ectodomain. The chimeric human THR-R/FSH-R has the variable pentapeptide sequence substituting for the native peptide sequence that is normally unmasked by thrombin action and constitutes the thrombin receptor agonist peptide, but it retains thrombin binding sequences and the thrombin specific cleavage site. Therefore, the amino terminus of expressed receptors is cleavable by thrombin at the appropriate location exposing a new amino terminus that is made of the variable pentapeptide segment of the library tethered to the transmembrane domains of hFSH-R.

To monitor for cell surface expression and efficient cleavage by thrombin of the amino terminal end of the chimeric receptors, an epitope-tag to which antibodies are available was positioned proximally to the thrombin cleavage site. Antibodies that recognize thrombin receptor amino terminus distal to the position corresponding to the library are also available. Consequently, chimeric receptors expressed on the cell surface are detectable by the appropriate use of both types of specific antibodies before thrombin treatment, but only with antibodies against the distal part after thrombin treatment.

The amino acid sequence of the chimeric human THR-R/FSH-R is shown in SEQ ID NO:2:

MDSKGSSQKGSRLLLLLVVSNLLLCQGVVSDYKDDDDKLDATLDPRXXXXXNPNDKYEPFWEDEEK
NESGLTEYRLVSINKSSPLQKQLPAFISEDASGYLGYNILRVLIWFISILAITGNIIVLVILTTSQ
YKLTVPRFLMCNLAFADLCIGIYLLLIASVDIHTKSQYHNYAIDWQTGAGCDAAGFFTVFASELSV
YTLTAITLERWHTITHAMQLDCKVQLRHAASVMVMGWIFAFAAALFPIFGISSYMKVSICLPMDID
SPLSQLYVMSLLVLNVLAFVVICGCYIHIYLTVRNPNIVSSSSDTRIAKRMAMLIFTDFLCMAPIS

PFAISASLKVPLITVSKAKILLVLFHPINSCANPFLYAIFTKNFRRDFFILLSKCGCYEMQAQIYR
TETSSTVHNTHPRNGHCSSAPRVTNGSTYILVPLSHLAQN

The construct consists of 436 amino acids: amino acid residues 1-30 represent the prolactin signal peptide (SEQ ID NO:6:MDSKGSSQKGSRLLLLLVVSNLLLCQGVVS); residues 31-38 represent the FLAG epitope (SEQ ID NO:4:DYKDDDDK); residues 39-101 represent amino acids from the hTHR receptor of which residues 47-51 represent the pentapeptide (SEQ ID NO:5:XXXXX) and residues 57-74 represent the hirudin epitope; and residues 102-436 represent amino acids from the hFSH receptor of which residues 108-128 represent transmembrane domain 1, residues 140-162 represent transmembrane domain 2, residues 185-206 represent transmembrane domain 3, residues 227-250 represent transmembrane domain 4, residues 270-291 represent transmembrane domain 5, residues 316-338 represent transmembrane domain 6, and residues 350-371 represent transmembrane domain 7. The signal peptide cleavage site lies between amino acid residues 30 and 31 of SEQ ID NO:2, and the thrombin cleavage site lies between amino acid residues 46 and 47 of SEQ ID NO:2. Cleavage with thrombin thus exposes the pentapeptide that is amino acid residues 47-51 of SEQ ID NO:2.

The construction of the DNA sequence encoding the amino acid sequence shown in SEQ ID NO:2 took several steps that are described below:

1) Construction of a sequence encoding the prolactin signal peptide (SEQ ID NO:3) followed by a FLAG epitope-tag (SEQ ID NO:4: DYKDDDDK) placed immediately upstream of the putative mature sequence for human thrombin receptor amino terminus ectodomain (from amino acids 34 to 95, SEQ ID NO:1) was produced by gene synthesis using standard techniques as described (Nussenzveig 1994). Synthetic oligonucleotides obtained for the prolactin leader sequence-FLAG epitope-tag construction have the

following sequences: coding strand PROLAC-1: SEQ ID NO:6: 5'- AAT TCC ACC ATG GAC TCC AAG GGC TCG AGC CAG AAG GGA TCT AGA CTG CT -3' ; complementary strand PROLAC-2: SEQ ID NO:7: 5'- PO_4- CAG CAG CAG TCT AGA TCC CTT CTG GCT CGA GCC CTT GGA GTC CAT GGT GG -3' ; coding strand PROLAC-3: SEQ ID NO:8: 5'- PO_4- G CTG CTG CTG GTG GTG AGC AAC CTG CTG CTG TGC CAG GGC GTC GTG -3' ; complementary strand PROLAC-4: SEQ ID NO:9: 5'- PO_4- CGC TCA CGA CGC CCT GGC ACA GCA GCA GGT TGC TCA CCA CCA GCA G - 3' ; FLAG-SENSE: SEQ ID NO:10: 5'- PO_4- AGC GAC TAC AAG GAC GAC GAC GAC AAG CTT CCT GCC TTT T -3' ; FLAG-ANTI-SENSE: SEQ ID NO:11: 5'- CGA AAA GGC AGG AAG CTT GTC GTC GTC GTC CTT GTA GT -3'. The pair of oligonucleotides PROLAC-1/PROLAC-2; PROLAC-3/PROLAC-4; and FLAG-SENSE/FLAG-ANTI-SENSE were annealed separately at 20 μM final oligonucleotide concentration, by heating at 95 °C for 5 min and cooling to 4 °C at a rate of 1 °C every 3 min, in 20 mM Tris-Cl pH 7.6 and 10 mM $MgCl_2$ buffer, using a thermal controller apparatus. Double stranded DNA was purified by agarose gel electrophoresis using the Mermaid™ purification system (Bio 101). Purified double stranded oligonucleotides were ligated using equal molar concentrations. Ligation products were digested with *Hind*III after heat inactivation of T4 DNA ligase. The resulting 125 bp larger fragment was purified by agarose gel electrophoresis using the Mermaid™ kit. Fragment of interest was subcloned into *Eco*RI and *Hind*III sites of pBSSKII(+). Correctness of the sequence was verified by dideoxynucleotide sequencing method using Circumvent sequencing kit (New England Biolabs, Inc.).

2) Construction of a sequence encoding the human thrombin receptor amino terminus from amino acid residue F^{55} to L^{96} (residues 60-101 of SEQ ID NO:2) was obtained by assembling four synthetic overlapping oligonucleotides containing gaps from 10 to 33 nucleotides: coding strand THRR-1: SEQ ID NO:12: 5' -

TAT GCC ACC TTT TGG GAG GAT GAG GAG AAA AAT GAA AGT GGG TTA ACT GAA TAC - 3'; complementary strand THRR-2: SEQ ID NO:13: 5' - TG AAG AGG ACT GCT TTT ATT GAT GGA GAC TAA TCT GTA TTC AGT TAA CCC ACT TTC - 3'; coding strand THRR-3: SEQ ID NO:14: 5' - C AAT AAA AGC AGT CCT CTT CAA AAA CAA CTT CCT GCA TTC ATC TCA GAA GAT GCC - 3'; complementary strand THRR *Bst*EII : SEQ ID NO:15: 5' - GT CAG GTA ACC GGA GGC ATC TTC TGA GAT GAA TGC AAG - 3'. Oligonucleotide THRR *Bst*EII inadvertently mutated hTHRR codon for P^{85} into L. Oligonucleotides THRR-2 and THRR-3 were phosphorylated enzymaticaly using T4 polynucleotide kinase in 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP and 25 μg/ml BSA. Oligonucleotides THRR-1, THRR-2, THRR-3 and THRR *Bst*EII were annealed at a final concentration of 10 μM in 20 mM Tris-Cl pH 7.6, 10 mM $MgCl_2$ buffer by heating at 95 °C for 5 min and cooling to 4 °C at a rate of 1 °C per 8 min using a thermal controller apparatus. The gaps between annealed oligonucleotides were filled-in using T4 DNA polymerase. Reaction was performed at a final concentration of 2.5 μM oligonucleotides, 400 μM dNTPs, 50 mM NaCl, 15 mM Tris-HCl, 12.5 mM $MgCl_2$, 1 mM dithiothreitol, 50 μg/ml BSA, pH 7.9 at 25 °C for 60 min. Reaction was stopped with 25 mM EDTA and enzyme was heat inactivated at 65 °C for 60 min. T4 DNA polymerase was selected not only to avoid strand displacement of the overlapping oligonucleotides but also because of its 3' to 5' exonuclease activity to correct the inadvertent mutation P^{85} to L.

3) Construction of a nucleotide sequence encoding amino acid residues from G^{361} to N^{694} (residues 102-436 of SEQ ID NO:2) followed by the stop codon of the hFSHR was obtained by standard *taq* polymerase PCR method using the following pair of oligonucleotides: i) coding strand FSHR *Bst*EII: SEQ ID NO:16: 5' - T GAA GGT TAC CTG GGG TAC AAC ATC CTC AGA GTC C - 3'; and ii) complementary strand *Not*I 2170: SEQ ID NO:17: 5' - TCA

CGC GGC CGC TTA GTT TTG GGC TAA ATG ACT TAG AGG - 3'. The resultant PCR product creates *Bst*EII and *Not*I sites at the 5' and 3' ends of the coding strand, respectively. The *Bst*EII site was used to connect the hFSHR sequence in frame with the amino terminus ectodomain of the hTHRR. The *Not*I site was used to connect the chimeric construct to the expression vector. Resulting PCR product was cloned into a pBSSKII(+)AT vector prepared to receive PCR fragments containing non-template dependent addition of 3' A-overhangs. pBSSKII(+)AT vector, that contains 3' T-overhangs, was obtained by ligating phosphorylated oligonucleotides AT-SENSE: SEQ ID NO:18: 5' - PO_4 AAT TCG GCT T - 3' and AT-ANTI-SENSE: SEQ ID NO:19: 5' - PO_4 AGC CG - 3' into pBSSKII(+) vector cut with *Eco*RI. A clone was selected with the orientation that places the newly created *Bst*EII site closer to the *Sac*I site of the vector and the *Not*I site of the insert closer to the *Kpn*I site of the vector.

4) Modification of the hFSHR construct obtained in item # 3 through the production of silent mutations to destroy the two *Pfl*MI sites originally present at positions 1,379 and 2,080 of the hFSHR cDNA. hFSHR DNA sequence was modified by PCR mutagenesis, using the construct obtained in item # 3 as a template in a standard PCR reaction with *taq* polymerase and the following three pairs of primers: i) *Pvu*I 1379 - ANTI-SENSE: SEQ ID NO:20: 5' - CA GTC GAT CGC ATA GTT GTG ATA TTG GCT C - 3' and vector REVERSE PRIMER: SEQ ID NO:21: 5' - AAC AGC TAT GAC CAT G - 3'. This PCR fragment contains a silent mutation that at the same time destroys a *Pfl*MI site present at position 1379 of the human FSHR cDNA and introduces a *Pvu*I site at the same position. ii) *Sac*II 2080 - SENSE: SEQ ID NO:22: 5' - C CAT CCG CGG AAT GGC CAC TGC TCT TCA GC - 3' and vector M13 (-20) PRIMER: SEQ ID NO:23: 5' - GTA AAA CGA CGG CCA GT - 3'. This PCR fragment contains a silent

mutation that at the same time destroys a *Pfl*MI site present at position 2,080 of the human FSHR cDNA and introduces a *Sac*II site at the same position. iii) *Pvu*I 1379 - SENSE: SEQ ID NO:24: 5' - TAT GCG ATC GAC TGG CAA ACT GGG GCA GG - 3' and *Sac*II 2080 - ANTI-SENSE: SEQ ID NO:25: 5' - C ATT CCG CGG ATG GGT GTT GTG GAC AGT G - 3'. PCR fragment that contains two silent mutations that simultaneously change a *Pfl*MI site present at positions 1,379 into a *Pvu*I site and a *Pfl*MI site present at position 2,080 into a *Sac*II site (numbers refer to the nucleotide sequence of the original hFSHR cDNA). PCR fragments originated with oligonucleotide pairs: i) was cut with the restriction enzymes *Bst*EII and *Pvu*I and a 225 bp DNA fragment was purified by agarose gel electrophoresis followed by the GeneClean™ procedure; ii) was cut with the restriction enzymes *Sac*II and *Apa*I and a 700 bp DNA fragment was purified by agarose gel electrophoresis followed by the GeneClean™ procedure; iii) was cut with the restriction enzymes *Pvu*I and *Sac*II and a 140 bp DNA fragment was purified by agarose gel electrophoresis followed by the Mermaid™ procedure. Construct obtained in step # 3 was cut with the restriction enzymes *Bst*EII and *Apa*I and a 2.9 kbp DNA fragment was purified by agarose gel electrophoresis followed by the GeneClean™ procedure. Finally the four purified DNA fragments were ligated together to produce a modified hFSHR construct in pBSSKII(+)AT vector.

5) Assembling of the hTHRR amino terminus ectodomain obtained from step # 2 with the modified hFSHR construct obtained from step # 4: the DNA fragment encoding the hTHRR amino terminus from amino acid residue F^{55} to L^{96} obtained from step # 2 was digested with the restriction enzyme *Bst*EII; the modified pBSSKII(+)AT-hFSHR construct obtained from step # 4 was linearized with the restriction enzyme *Sac*I and blunted with T4 DNA polymerase to remove 3'

overhangs. After enzyme inactivation, blunted linear pBSSKII(+)AT-hFSHR was cut with the restriction enzyme *Bst*EII. After appropriate DNA fragment purification the two modified DNA fragments (hTHRR Blunt-*Bst*EII ~130 bp long and pBSSKII(+)AT-hFSHR Blunt-*Bst*EII ~3,800 bp long) generated at this step (# 5) were ligated together to form the intermediate construct pBSSKII(+)hTHRR/hFSHR. Correctness of the sequence was verified by dideoxynucleotide sequencing method using Circumvent sequencing kit (New England Biolabs, Inc.).

6) Plasmid construct generated at step # 1 (pBSSKII(+) PROLAC FLAG (*Eco*RI-*Hind*III)) was cut with the restriction enzymes *Hind*III and *Apa*I and the larger fragment was purified by the GeneClean™ procedure. Plasmid construct obtained at step # 6 (intermediate pBSSKII(+)hTHRR/hFSHR) was cut with the restriction enzymes *Pfl*MI and *Apa*I and the resulting ~1,100 bp DNA fragment was purified by agarose gel electrophoresis followed by the GeneClean™ procedure. A pair of oligonucleotides CONNECT-SENSE: SEQ ID NO:26: 5′ - AG CTT GAT GCC ACG CTA TGG CCC TAG GTA AGT GAT ATG CCA CCT T - 3′; and CONNECT-ANTI-SENSE: SEQ ID NO:27: 5′ - G TGG CAT ATC ACT TAC CTA GGG CCA TAG CGT GGC ATC A - 3′ were annealed and purified using the procedure described in step # 1. Annealed CONNECT-SENSE/CONNECT-ANTI-SENSE oligonucleotides were used to adapt the overhang created by *Hind*III digestion of pBSSKII(+) PROLAC FLAG with the overhang created by *Pfl*MI digestion of intermediate pBSSKII(+)hTHRR/hFSHR, both mentioned above. Therefore, a ligation of the two DNA fragments purified above was performed using the adaptor CONNECT-SENSE/CONNECT-ANTI-SENSE. This adaptor not only regenerates the two restriction enzyme sites, *Hind*III and *Pfl*MI, but also introduces a second *Pfl*MI site between the *Hind*III and the first *Pfl*MI sites. *Pfl*MI restriction enzyme recognition sequence is an interrupted palindrome that allows for the directional

clone of DNA fragments with appropriate overhangs. The two *Pfl*MI restriction enzyme sites were created to subclone a synthetic DNA fragment that introduces, in frame with both the prolactin leader sequence/FLAG epitope tag and the chimeric hTHRR/hFSHR sequence described in step # 5, the variable pentapeptide agonist library preceded and followed by hTHRR amino acid residues corresponding to hTHRR residues from L^{34} to P^{55}. CONNECT-SENSE/CONNECT-ANTI-SENSE adaptor sequence introduces stop codons at each of the three possible reading frames to decrease background levels during the construction of the library. Correctness of the sequence was verified by dideoxynucleotide sequencing method using automatic sequencing.

7) Subcloning of the construct obtained at step # 6 into the mammalian expression vector pcDNA3 and into unmodified pBSSKII(+). Plasmid obtained in step # 6 was digested with *Eco*RI and *Not*I, followed by purification of the ~1300 bp DNA insert. pcDNA3 and pBSSKII(+) were both prepared by cutting with *Eco*RI and *Not*I. Insert and vectors were ligated. A library created in the vector pcDNA3 is used in transfection experiments using mammalian cells. Fig. 9 shows the plasmid map for the THRR/FSHR construct designated pcDNA3PROLACFLAGhTHRR/hFSHR. A library created in the vector pBSSKII(+) is used for *in vitro* transcription after linearization with the restriction enzyme *Not*I. Resulting RNAs are injected into *Xenopus* oocytes.

8) Library construction: three oligonucleotides: coding strand LIBRARY-1: SEQ ID NO:28: 5' - PO_4 - A GAT CCC CGG NNS NNS NNS NNS NNS AAC CCC AAT GAT AAA TAT GAA CCC TT - 3', where N means all four nucleotides and S means either G or C, a degenerate oligonucleotide pool with 2^{25} different nucleic acid molecules, encoding 20^5 different pentapeptide sequences; complementary strand LIBRARY-2: SEQ ID NO:29: 5' - PO_4 - CCG GGG ATC TAG C - 3'; and complementary strand LIBRARY-3: SEQ ID NO:30:

5' - PO_4 - GG TTC ATA TTT ATC - 3' were annealed at a molar ratio of 1 (LIBRARY-1) : 25 (LIBRARY-2) : 25 (LIBRARY-3) in 20 mM Tris-HCl pH 7.6 , 10 mM $MgCl_2$, by heating at 95 °C for 5 min and cooling to 4 °C at a rate of 1 °C per 8 min using a thermal controller apparatus. Annealed oligonucleotides were purified by agarose gel electrophoresis using the Mermaid™ kit. pcDNA3 PROLACFLAG-CONNECT-SENSE/CONNECT-ANTI-SENSE-hTHRR/hFSHR or pBSSKII(+)PROLACFLAG-CONNECT-SENSE/CONNECT-ANTI-SENSE-hTHRR/hFSHR from step # 7 were cut with the restriction enzyme *Pfl*MI to completion. Purified large fragment of each construct was ligated with the annealed library oligonucleotides at approximately 1:3 molar ratios. The ligated products were ethanol precipitated and redissolved in water for transformation of *E. coli* XL1-Blue cells by electroporation.

Shown below is the nucleotide sequences of PROLAC FLAG - CONNECT-SENSE/CONNECT- ANTI-SENSE - hTHRR/hFSHR and of the assembled LIBRARY-1,-2 and -3 oligonucleotides to be ligated into the two *Pfl*MI sites of the insert, substituting for most of the sequence corresponding to CONNECT-SENSE/CONNECT-ANTI-SENSE:

```
Eco R I AATTCCACC ATG GAC TCC AAG GGC TCG AGC CAG AAG
              GGTGG TAC CTG AGG TTC CCG AGC TCG GTC TTC
                     M   D   S   K   G   S   S   Q   K

GGA TCT AGA CTG CTG CTG CTG CTG GTG GTG AGC AAC CTG CTG
CCT AGA TCT GAC GAC GAC GAC GAC CAC CAC TCG TTG GAC GAC
 G   S   R   L   L   L   L   L   V   V   S   N   L   L

CTG TGC CAG GGC GTC GTG AGC GAC TAC AAG GAC GAC GAC GAC
GAC ACG GTC CCG CAG CAC TCG CTG ATG TTC CTG CTG CTG CTG
 L   C   Q   G   V   V   S   D   Y   K   D   D   D   D

  A Hind III AG CTT GAT GCC ACG CT Pfl M I A TGG CCC TAG
TTC GA           A CTA CGG TG           C GAT ACC GGG ATC
              K  L   D   A   T   L                      *

GTA AGT GAT ATG CCAC C TT  Pfl M I T TGG GAG GAT GAG GAG
CAT TCA CTA TAC GG             G AAA ACC CTC CTA CTC CTC
 *       *                        F   W   E   D   E   E

AAA AAT CAA AGT GGG TTA ACT GAA TAC AGA TTA GTC TCC ATC
TTT TTA CTT TCA CCC AAT TGA CTT ATG TCA AAT CAG AGG TAG
 K   N   E   S   G   L   T   E   Y   R   L   V   S   I
```

```
AAT AAA AGC AGT CCT CTT CAA AAA CAA CTT CCT GCA TTC ATC
TTA TTT TCG TCA GGA GAA GTT TTT GTT GAA GGA CGT AAG TAG
 N   K   S   S   P   L   Q   K   Q   L   P   A   F   I

TCA GAA GAT GCC TCC G BstE II GT TAC CTG GGG TAC AAC
AGT CTT CTA CGG AGG CCA ATG         GAC CCC ATG TTG
 S   E   D   A   S              G   Y   L   G   Y   N

ATC CTC AGA GTC CTG ATA TGG TTT ATC AGC ATC CTG GCC ATC
TAG GAG TCT CAG GAC TAT ACC AAA TAG TCG TAG GAC CGG TAG
 I   L   R   V   L   I   W   F   I   S   I   L   A   I

ACT GGG AAC ATC ATA GTG CTA GTG ATC CTA ACT ACC AGC CAA
TGA CCC TTG TAG TAT CAC GAT CAC TAG GAT TGA TGG TCG GTT
 T   G   N   I   I   V   L   V   I   L   T   T   S   Q

TAT AAA CTC ACA GTC CCC AGG TTC CTT ATG TGC AAC CTG GCC
ATA TTT GAG TGT CAG GGG TCC AAG GAA TAC ACG TTG GAC CGG
 Y   K   L   T   V   P   R   F   L   M   C   N   L   A

TTT GCT GAT CTC TGC ATT GGA ATC TAC CTG CTG CTC ATT GCA
AAA CGA CTA GAG ACG TAA CCT TAG ATG GAC GAC GAG TAA CGT
 F   A   D   L   C   I   G   I   Y   L   L   L   I   A

TCA GTT GAT ATC CAT ACC AAG AGC CAA TAT CAC AAC TAT GCG
AGT CAA CTA TAG GTA TGG TTC TCG GTT ATA GTG TTG ATA CGC
 S   V   D   I   H   T   K   S   Q   Y   H   N   Y   A

AT PvuI C GAC TGG CAA ACT GGG GCA GGC TGT GAT GCT GCT
        TAG CTG ACC GTT TGA CCC CGT CCG ACA CTA CGA CGA
 I           D   W   Q   T   G   A   G   C   D   A   A

GGC TTT TTC ACT GTC TTT GCC AGT GAG CTG TCA GTC TAC ACT
CCG AAA AAG TGA CAG AAA CGG TCA CTC GAC AGT CAG ATG TGA
 G   F   F   T   V   F   A   S   E   L   S   V   Y   T

CTG ACA GCT ATC ACC TTG GAA AGA TGG CAT ACC ATC ACG CAT
GAC TGT CGA TAG TGG AAC CTT TCT ACC GTA TGG TAG TGC GTA
 L   T   A   I   T   L   E   R   W   H   T   I   T   H

GCC ATG CAG CTG GAC TGC AAG GTG CAG CTC CGC CAT GCT GCC
CGG TAC GTC GAC CTG ACG TTC CAC GTC GAG GCG GTA CGA CGG
 A   M   Q   L   D   C   K   V   Q   L   R   H   A   A

AGT GTC ATG GTG ATG GGC TGG ATT TTT GCT TTT GCA GCT GCC
TCA CAG TAC CAC TAC CCG ACC TAA AAA CGA AAA CGT CGA CGG
 S   V   M   V   M   G   W   I   F   A   F   A   A   A

CTC TTT CCC ATC TTT GGC ATC AGC AGC TAC ATG AAG GTG AGC
GAG AAA GGG TAG AAA CCG TAG TCG TCG ATG TAC TTC CAC TCG
 L   F   P   I   F   G   I   S   S   Y   M   K   V   S

ATC TGC CTG CCC ATG GAT ATT GAC AGC CCT TTG TCA CAG CTG
TAG ACG GAC GGG TAC CTA TAA CTG TCG GGA AAC AGT GTC GAC
 I   C   L   P   M   D   I   D   S   P   L   S   Q   L

TAT GTC ATG TCC CTC CTT GTG CTC AAT GTC CTG GCC TTT GTG
ATA CAG TAC AGG GAG GAA CAC GAG TTA CAG GAC CGG AAA CAC
 Y   V   M   S   L   L   V   L   N   V   L   A   F   V

GTC ATC TGT GGC TGC TAT ATC CAC ATC TAC CTC ACA GTG CGG
CAG TAG ACA CCG ACG ATA TAG GTG TAG ATG GAG TGT CAC GCC
 V   I   C   G   C   Y   I   H   I   Y   L   T   V   R

AAC CCC AAC ATC GTG TCC TCC TCT AGT GAC ACC AGG ATC GCC
TTG GGG TTG TAG CAC AGG AGG AGA TCA CTG TGG TCC TAG CGG
 N   P   N   I   V   S   S   S   D   T   R   I   A
```

```
AAG CGC ATG GCC ATG CTC ATC TTC ACT GAC TTC CTC TGC ATG
TTC GCG TAC CGG TAC GAG TAG AAG TGA CTG AAG GAG ACG TAC
 K   R   M   A   M   L   I   F   T   D   F   L   C   M

GCA CCC ATT TCT TTC TTT GCC ATT TCT GCC TCC CTC AAG GTG
CGT GGG TAA AGA AAG AAA CGG TAA AGA CGG AGG GAG TTC CAC
 A   P   I   S   F   F   A   I   S   A   S   L   K   V

CCC CTC ATC ACT GTG TCC AAA GCA AAG ATT CTG CTG GTT CTG
GGG GAG TAG TGA CAC AGG TTT CGT TTC TAA GAC GAC CAA GAC
 P   L   I   T   V   S   K   A   K   I   L   L   V   L

TTT CAC CCC ATC AAC TCC TGT GCC AAC CCC TTC CTC TAT GCC
AAA GTG GGG TAG TTG AGG ACA CGG TTG GGG AAG GAG ATA CGG
 F   H   P   I   N   S   C   A   N   P   F   L   Y   A

ATC TTT ACC AAA AAC TTT CGC AGA GAT TTC TTC ATT CTG CTG
TAG AAA TGG TTT TTG AAA GCG TCT CTA AAG AAG TAA GAC GAC
 I   F   T   K   N   F   R   R   D   F   F   I   L   L

AGC AAG TGT GGC TGC TAT GAA ATG CAA GCC CAA ATT TAT AGG
TCG TTC ACA CCG ACG ATA CTT TAC GTT CGG GTT TAA ATA TCC
 S   K   C   G   C   Y   E   M   Q   A   Q   I   Y   R

ACA GAA ACT TCA TCC ACT GTC CAC AAC ACC CAT CCG C Sac
TGT CTT TGA AGT AGG TGA CAG GTG TTG TGG GTA GG
 T   E   T   S   S   T   V   H   N   T   H   P

II GG AAT GGC CAC TGC TCT TCA GCT CCC AGA GTC ACC AAT
C GCC TTA CCG GTG ACG AGA AGT CGA GGG TCT CAG TGG TTA
   R   N   G   H   C   S   S   A   P   R   V   T   N

GGT TCC ACT TAC ATA CTT GTC CCT CTA AGT CAT TTA GCC CAA
CCA AGG TGA ATG TAT GAA CAG GGA GAT TCA GTA AAT CGG GTT
 G   S   T   Y   I   L   V   P   L   S   H   L   A   Q

AAC TAA GC Not I
TTG ATT CGCCGG
 N   *
```

The DNA sequence (sense strand) is shown in SEQ ID NO:31, with the antisense strand shown in SEQ ID NO:32. The DNA sequence is the sequence that would encode the amino acid sequence (SEQ ID NO:33) of the chimeric preprotein that is prolactin signal peptide/FLAG epitope tag/hTHR receptor amino terminus corresponding to amino acid residues 34 to 96 in the native receptor/hFSH receptor. There are three nonsense ("stop") codons (one in each potential reading frame) in the middle of the sequence encoding the hTHR receptor amino terminus that are present to prevent translation of this precursor sequence if this sequence persisted, that is remained uncut, during construction of the final library (see below). These "stop" codons, therefore, would prevent translation of non-recombinant

protein. To construct the library, this sequence (SEQ ID NO:31) is cut with PflMI to excise one small DNA fragment flanked by two PflMI restriction sites that is replaced with the following DNA sequences: sense, SEQ ID NO:28; antisense, SEQ ID NO:29 and SEQ ID NO:30) that encodes the pentapeptide library (amino acid SEQ ID NO:42):

```
Pfl M I A GAT CCC CGG NNS NNS NNS NNS NNS AAC CCC AAT
        C GAT CTA GGG GCC
   T   L   D   P   R   X   X   X   X   X   N   P   N

GAT AAA TAT GAA CCC TT Pfl M I
CTA TTT ATA CTT GG
 D   K   Y   E   P   F
```

Modification of the original construct with the intent to create a combinatorial peptide library that expresses random pentapeptides tethered to the seven transmembrane helical bundle of any GPCR already in an "active" or "exposed" form, without the need for cleavage by thrombin. Use of the human follicle stimulating hormone receptor (hFSH-R) as the initial library construction:

In this version of the library, the variable pentapeptide sequence is placed immediately after the prolactin signal peptide. Consequently, the cleavage produced by the signal peptidase that normally occurs during synthesis of type III membrane proteins would expose or "activate" the pentapeptide present at the beginning of the amino terminus, allowing it to interact with the seven transmembrane helical bundle of the GPCR to which it is tethered. The resulting protein sequence of the amino terminus ectodomain that can replace the amino terminus ectodomain of any GPCR is shown in SEQ ID NO:34:

```
MDSKGSSQKGSRLLLLLVVSNLLLCQGVVSXXXXXNPNDKYEPFWEDEEKNESGLTEYRLVSINKS
SPLQKQLPAFISEDASGYL
```

To create this construct it is necessary to perform a small modification in the construct already obtained for the discovery of peptide agonists for the hFSHR using thrombin activation. By silent mutation using PCR method, it is possible to introduce a new *Pfl*MI restriction endonuclease cleavage site in the sequence of prolactin signal peptide construct obtained previously at step # 1: PCR using the pair of oligonucleotide primers : complementary strand *Pfl*MI SIGNAL: SEQ ID NO:35: 5' - ATC AAG CTT GTC GTC GTC GTC CTT GTA GTC GCT CAC <u>C</u>AC GCC CTG - 3' and vector M13 (-20) PRIMER: SEQ ID NO:23: 5' - GTA AAA CGA CGG CCA GT - 3', using as template the construct pBSSKII(+) PROLAC FLAG - CONNECT-SENSE/CONNECT-ANTI-SENSE - hTHRR/hFSHR. Resulting PCR product is cut with *Eco*RI and *Hind*III. This fragment will substitute the corresponding fragment in pBSSKII(+) PROLAC FLAG - CONNECT-SENSE/CONNECT-ANTI-SENSE - hTHRR/hFSHR, therefore introducing a third *Pfl*MI restriction enzyme recognition site at the desired position. See sequence below (sense, SEQ ID NO:36; antisense, SEQ ID NO:37; amino acid, SEQ ID NO:38):

```
Eco R I  AATTCCACC ATG GAC TCC AAG GGC TCG AGC CAG AAG
              GGTGG TAC CTG AGG TTC CCG AGC TCG GTC TTC
                     M   D   S   K   G   S   S   Q   K

GGA TCT AGA CTG CTG CTG CTG CTG GTG GTG AGC AAC CTG CTG
CCT AGA TCT GAC GAC GAC GAC GAC CAC CAC TCG TTG GAC GAC
 G   S   R   L   L   L   L   L   V   V   S   N   L   L

CTG TGC CAG GGC  Pfl M I GTG GTG AGC GAC TAC AAG GAC GAC
GAC ACG GTC          CCG CAC CAC TCG CTG ATG TTC CTG CTG
 L   C   Q   G            V   V   S   D   Y   K   D   D

GAC GAC A Hind III   AG CTT GAT GCC ACG CT  Pfl M I  A TGG
CTG CTG TTC GA            A CTA CGG TG             C GAT ACC
 D   D                K   L   D   A   T   L              L   W

CCC TAG GTA AGT GAT ATG CCAC C TT Pfl M I  T TGG GAG GAT
GGG ATC CAT TCA CTA TAC GGTG              G AAA ACC CTC CTA
 P   *   *   *                              F   W   E   D

GAG GAG AAA AAT GAA AGT GGG TTA ACT GAA TAC AGA TTA GTC TCC
CTC CTC TTT TTA CTT TCA CCC AAT TGA CTT ATG TCA AAT CAG AGG
 E   E   K   N   E   S   G   L   T   E   Y   R   L   V   S

ATC AAT AAA AGC AGT CCT CTT CAA AAA CAA CTT CCT GCA TTC ATC
```

```
TAG TTA TTT TCG TCA GGA GAA GTT TTT GTT GAA GGA CGT AAG TAG
 I   N   K   S   S   P   L   Q   K   Q   L   P   A   F   I

TCA GAA GAT GCC TCC G Bst E II GT TAC CTG GGG TAC AAC
AGT CTT CTA CGG AGG CCA ATG           GAC CCC ATG TTG
 S   E   D   A   S           G   Y   L   G   Y   N

ATC CTC AGA GTC CTG ATA TGG TTT ATC AGC ATC CTG GCC ATC
TAG GAG TCT CAG GAC TAT ACC AAA TAG TCG TAG GAC CGG TAG
 I   L   R   V   L   I   W   F   I   S   I   L   A   I

ACT GGG AAC ATC ATA GTG CTA GTG ATC CTA ACT ACC AGC CAA
TGA CCC TTG TAG TAT CAC GAT CAC TAG GAT TGA TGG TCG GTT
 T   G   N   I   I   V   L   V   I   L   T   T   S   Q

TAT AAA CTC ACA GTC CCC AGG TTC CTT ATG TGC AAC CTG GCC
ATA TTT GAG TGT CAG GGG TCC AAG GAA TAC ACG TTG GAC CGG
 Y   K   L   T   V   P   R   F   L   M   C   N   L   A

TTT GCT GAT CTC TGC ATT GGA ATC TAC CTG CTG CTC ATT GCA
AAA CGA CTA GAG ACG TAA CCT TAG ATG GAC GAC GAG TAA CGT
 F   A   D   L   C   I   G   I   Y   L   L   L   I   A

TCA GTT GAT ATC CAT ACC AAG AGC CAA TAT CAC AAC TAT GCG
AGT CAA CTA TAG GTA TGG TTC TCG GTT ATA GTG TTG ATA CGC
 S   V   D   I   H   T   K   S   Q   Y   H   N   Y   A

AT PvuI C GAC TGG CAA ACT GGG GCA GGC TGT GAT GCT GCT
      TAG CTG ACC GTT TGA CCC CGT CCG ACA CTA CGA CGA
 I        D   W   Q   T   G   A   G   C   D   A   A

GGC TTT TTC ACT GTC TTT GCC AGT GAG CTG TCA GTC TAC ACT
CCG AAA AAG TGA CAG AAA CGG TCA CTC GAC AGT CAG ATG TGA
 G   F   F   T   V   F   A   S   E   L   S   V   Y   T

CTG ACA GCT ATC ACC TTG GAA AGA TGG CAT ACC ATC ACG CAT
GAC TGT CGA TAG TGG AAC CTT TCT ACC GTA TGG TAG TGC GTA
 L   T   A   I   T   L   E   R   W   H   T   I   T   H

GCC ATG CAG CTG GAC TGC AAG GTG CAG CTC CGC CAT GCT GCC
CGG TAC GTC GAC CTG ACG TTC CAC GTC GAG GCG GTA CGA CGG
 A   M   Q   L   D   C   K   V   Q   L   R   H   A   A

AGT GTC ATG GTG ATG GGC TGG ATT TTT GCT TTT GCA GCT GCC
TCA CAG TAC CAC TAC CCG ACC TAA AAA CGA AAA CGT CGA CGG
 S   V   M   V   M   G   W   I   F   A   F   A   A   A

CTC TTT CCC ATC TTT GGC ATC AGC AGC TAC ATG AAG GTG AGC
GAG AAA GGG TAG AAA CCG TAG TCG TCG ATG TAC TTC CAC TCG
 L   F   P   I   F   G   I   S   S   Y   M   K   V   S

ATC TGC CTG CCC ATG GAT ATT GAC AGC CCT TTG TCA CAG CTG
TAG ACG GAC GGG TAC CTA TAA CTG TCG GGA AAC AGT GTC GAC
 I   C   L   P   M   D   I   D   S   P   L   S   Q   L

TAT GTC ATG TCC CTC CTT GTG CTC AAT GTC CTG GCC TTT GTG
ATA CAG TAC AGG GAG GAA CAC GAG TTA CAG GAC CGG AAA CAC
 Y   V   M   S   L   L   V   L   N   V   L   A   F   V

GTC ATC TGT GGC TGC TAT ATC CAC ATC TAC CTC ACA GTG CGG
CAG TAG ACA CCG ACG ATA TAG GTG TAG ATG GAG TGT CAC GCC
 V   I   C   G   C   Y   I   H   I   Y   L   T   V   R

AAC CCC AAC ATC GTG TCC TCC TCT AGT GAC ACC AGG ATC GCC
TTG GGG TTG TAG CAC AGG AGG AGA TCA CTG TGG TCC TAG CGG
 N   P   N   I   V   S   S   S   S   D   T   R   I   A
```

```
AAG CGC ATG GCC ATG CTC ATC TTC ACT GAC TTC CTC TGC ATG
TTC GCG TAC CGG TAC GAG TAG AAG TGA CTG AAG GAG ACG TAC
 K   R   M   A   M   L   I   F   T   D   F   L   C   M

GCA CCC ATT TCT TTC TTT GCC ATT TCT GCC TCC CTC AAG GTG
CGT GGG TAA AGA AAG AAA CGG TAA AGA CGG AGG GAG TTC CAC
 A   P   I   S   F   F   A   I   S   A   S   L   K   V

CCC CTC ATC ACT GTG TCC AAA GCA AAG ATT CTG CTG GTT CTG
GGG GAG TAG TGA CAC AGG TTT CGT TTC TAA GAC GAC CAA GAC
 P   L   I   T   V   S   K   A   K   I   L   L   V   L

TTT CAC CCC ATC AAC TCC TGT GCC AAC CCC TTC CTC TAT GCC
AAA GTG GGG TAG TTG AGG ACA CGG TTG GGG AAG GAG ATA CGG
 F   H   P   I   N   S   C   A   N   P   F   L   Y   A

ATG TTT ACC AAA AAC TTT CGC AGA GAT TTC TTC ATT CTG CTG
TAG AAA TGG TTT TTG AAA GCG TCT CTA AAG AAG TAA GAC GAC
 I   F   T   K   N   F   R   R   D   F   F   I   L   L

AGC AAG TGT GGC TGC TAT GAA ATG CAA GCC CAA ATT TAT AGG
TCG TTC ACA CCG ACG ATA CTT TAC GTT CGG GTT TAA ATA TCC
 S   K   C   G   C   Y   E   M   Q   A   Q   I   Y   R

ACA GAA ACT TCA TCC ACT GTC CAC AAC ACC CAT CCG C Sac
TGT CTT TGA AGT AGG TGA CAG GTG TTG TGG GTA GG
 T   E   T   S   S   T   V   H   N   T   H   P

II GG AAT GGC CAC TGC TCT TCA GCT CCC AGA GTC ACC AAT
C GCC TTA CCG GTG ACG AGA AGT CGA GGG TCT CAG TGG TTA
   R   N   G   H   C   S   S   A   P   R   V   T   N

GGT TCC ACT TAC ATA CTT GTC CCT CTA AGT CAT TTA GCC CAA
CCA AGG TGA ATG TAT GAA CAG GGA GAT TCA GTA AAT CGG GTT
 G   S   T   Y   I   L   V   P   L   S   H   L   A   Q

AAC TAA GC Not I
TTG ATT CGCCGG
 N   *
```

Two of the three LIBRARY oligonucleotides are also need to be modified as follows: coding strand LIBRARY-4: SEQ ID NO:39: 5′ - PO_4 - GTG GTG AGC NNS NNS NNS NNS NNS AAC CCC AAT GAT AAA TAT GAA CCC TT - 3′; and complementary strand LIBRARY-5: SEQ ID NO:40: 5′ - PO_4 - GCT CAC CAC GCC - 3′.

The nucleotide sequence of the assembled LIBRARY-4,-5 and -3 oligonucleotides to be ligated into the two *Pfl*MI sites of the insert, substituting for the sequence corresponding to FLAG - CONNECT-SENSE/ CONNECT-ANTI-SENSE is shown below (sense, SEQ ID NO:39; antisense, SEQ ID NO:40 and SEQ ID NO:30; amino acid, SEQ ID NO:41):

```
Pfl M I   GTG GTG AGC NNS NNS NNS NNS NNS AAC CCC AAT GAT
```

```
CCG CAC CAC TCG                                    CTA
 G   V   V   S   X   X   X   X   X   N   P   N   D

AAA TAT GAA CCC TT Pfl M I
TTT ATA CTT GG
 K   Y   E   P   F
```

The DNA sequence is shown in SEQ ID NO:39. This sequence is the same as SEQ ID NO:31 except that it has a "silent" mutation that creastes another PflMI restriction site. The DNA sequence (SEQ ID NO:39) is the sequence that would encode the amino acid sequence (SEQ ID NO:41) of the chimeric preprotein that is prolactin signal peptide/FLAG epitope tag/hTHR receptor amino terminus corresponding to amino acid residues 34 to 96 in the native receptor/hFSH receptor sequence from amino acid residue 361 to 694 of the native hFSH receptor. There are three nonsense ("stop") codons (one in each potential reading frame) in the middle of the sequence encoding the hTHR receptor amino terminus that are present to prevent translation of this precursor sequence if this sequence persisted, that is remained uncut, during construction of the final library. These "stop" codons, therefore, would prevent translation of non-recombinant protein. To construct the library, this sequence (SEQ ID NO:39) is cut with PflMI to excise two small DNA fragments flanked by two PflMI restriction sites that is replaced with the following DNA sequences: sense, SEQ ID NO:39; antisense, SEQ ID NO:40 and SEQ ID NO:30) that encodes the pentapeptide library (SEQ ID NO:41).

EXAMPLE II

Peptide Agonists of hCTR

Fig. 6 illustrates the putative two-dimensional topology of a human calcitonin receptor (hCTR). The top of the diagram represents the extracellular (EC) space, the middle portion represents the transmembrane (TM) domain, and the bottom portion represents the intracellular (IC) space. Each circle

represents a single amino acid residue designated by the single letter code. The residues specifically referred to in this application are numbered with regard to hCTR-2. Bold lines demarcate the 3 isoforms: hCTR-1 - all residues; hCTR-2 - missing the 16 amino acids between R174 and S175; and hCTR-3 - missing residues 1-47.

To discover small peptides that can serve as agonists for hCTR, a combinatorial peptide library was constructed that expresses random pentapeptides tethered to the seven TM helical bundle of hCTR. A pentapeptide library was chosen based on the fact that TRH is a tripeptide that is blocked at both ends (3+2 (for block) =5) and the resulting number of clones is workable. The constructed library contains all 20 natural amino acids at each of the five positions and therefore has a complexity of $20^5 = 3.2 \times 10^6$ possible combinations.

To this end, the complementary DNA (cDNA) sequence that normally encodes hCTR's N-terminal EC domain is substituted by a DNA sequence that encodes the thrombin receptor's N- terminal ectodomain. The chimeric ThrR/hCTR has the variable pentapeptide sequence substituting for the native peptide sequence that is normally unmasked by thrombin action and constitutes the ThrR peptide agonist, but it retains thrombin binding sequences and the thrombin-specific cleavage site. Therefore, the N-terminus of expressed receptors are cleaved by thrombin at the appropriate location exposing a new N-terminus that is made of the variable pentapeptide segment of the library tethered to the remainder of hCTR, that is, in a position that in the native ThrR allows it to serve as an agonist.

To monitor for cell surface expression and for efficient cleavage by thrombin of the N- terminal end of the chimeric receptors, the FLAG epitope is positioned proximally to the thrombin cleavage site.

Abs that recognize ThrR N-terminus distal to the position corresponding to the library are also used. Consequently, chimeric receptors expressed on the cell surface are detectable by the appropriate use of both Abs before thrombin treatment, but only with Abs against the distal part after thrombin treatment. This confirms cell-surface expression and adequacy of thrombin generation of potential agonists.

The cDNA sequence encoding the new N-terminus of the chimeric ThrR/hCTR, consisting of a prolactin leader or signal peptide, followed by the FLAG epitope, followed by the N- terminus of the mature human ThrR and the pentapeptide library is constructed by gene synthesis. It consists of a DNA segment of approximately 300 base pairs encoding 100 amino acids that is ligated in frame through an appropriate restriction endonuclease cleavage site that is in the synthetic hCTR-2 cDNA at a position encoding the amino acids that constitute the transition between the N-terminus and the first TM domain. After ligation into a mammalian expression vector, *Escherichia coli* is transformed by electroporation and the transformants are subdivided into pools whose maximal workable complexity is determined according to the efficiency of expression and/or sensitivity of the detection system.

The success of expression cloning strategies, such as the one of the subject invention, is dependent on the reporter (or detection) system used. An amplified reporter system is used in accordance with the subject invention which is based on the second messenger systems triggered by hCTR. hCTR is a GPCR that upon activation in COS-1 cells couples through the G protein, G_s, to the enzyme adenylyl cyclase, causing an increase in intracellular concentrations of cyclic AMP (cAMP) and through G_q to the enzyme phospholipase C causing increases in inositol 1,4,5-trisphosphate (IP3), which causes a rise in intracellular free Ca^{2+},

and 1,2-diacylglycerol (DAG) (Nussenzveig et al. 1994). cAMP activates cAMP- dependent protein kinase (PKA), an important intracellular regulator. One PKA substrate is a transcription factor known as cAMP-response element binding protein, CREB, that when activated binds and activates transcription by promoters that contain regulatory sequences known as cAMP-response elements (CREs). A similar cascade initiated by IP3, DAG and an elevation of cytoplasmic Ca^{2+} that involves other protein kinases triggers gene induction through other motifs using other transcription factors, such as the fos-jun- AP-1 system. This type of reporter system is able to detect basal as well as CT-stimulated activation of the wild type hCTR by using a reporter plasmid containing a minimal promoter of human c-*fos* gene into which a CREB binding motif (Montminy et al. 1990) was engineered driving transcription of the gene for the enzyme luciferase (pCRE/LUC), whose activity is easily detected by a chemiluminescent reaction. Unfortunately, the use of the enzyme activity of the luciferase reporter system requires the preparation of cell extracts and, therefore, monitors induction in a population of cells. To be able to measure a single positive in a very large number of negatives from a library, a single cell assay is needed. Two different assays are used so as to improve the likelihood of identifying positive clones.

One assay is based on gene induction in COS-1 cells. β-galactosidase is used as a reporter gene in transfected COS-1 cells. This assay takes advantage of the amplification of the enzyme activity of the reporter, with an easily determined color reaction as endpoint, and of the over-expression of receptors with tethered agonists in COS-1 cells because of replication of the plasmids introduced. These experiments were performed by co-transfecting portions of the library and CRE/β-galactosidase or AP-1/β-galactosidase

constructs into COS-1 cells so as to amplify expression by plasmid replication using the simian virus-40 origin of replication in the vector. This enhances the signal/noise ratio substantially. Both promoter types are used as hCTR-2 transduces signals by both pathways in COS-1 cells. The signal is further increased because the construct used has a nuclear localization signal ligated to the β-galactosidase that allows the protein to concentrate in the nucleus (Hersh et al. 1995). Single clones that exhibit activation of chimeric ThrR/hCTR after thrombin addition to cleave the N-terminus and expose the tethered agonist, as measured by increased color reaction, are isolated using sib selection, which consists of successive subdivision and amplification of positive pools of clones. The optimal time after thrombin addition to measure the reporter gene activity is determined, as this involves a prolonged response on gene induction and the kinetics of this response vary with different activators (receptors) and in different cells. The optimal time will likely be 4 hrs as that was optimal for sCT stimulation in COS-1 cells co-transfected with hCTR-2 and luciferase under the control of a cAMP-responsive promoter (CRE-LUC). Even though hCTR-2 exhibits constitutive activity, gene expression can be further activated with agonist.

The second reporter system uses *Xenopus laevis* oocytes. This system, which was used to clone the TRH receptor cDNA (Straub et al. 1990), is dependent on coupling to G_q. In this assay system, individual oocytes are injected with RNA that was transcribed *in vitro* from pools of plasmids from the library. After one to three days (the optimal time for responsiveness is determined in preliminary experiments with hCTR-2), the responsiveness of the oocytes to thrombin is measured. Thrombin acutely activates receptors in those oocytes expressing chimeric

constructs in which the peptide can serve as a tethered agonist. Receptor activation is monitored by acute effects on a chloride current or on stimulation of radiocalcium efflux. Both endpoints are rapid, amplified processes in oocytes. Single clones that exhibit activation of chimeric ThrR/hCTR after thrombin addition are isolated using sib selection. Using both reporter systems allows the determination of whether rapid or more prolonged effects yield better signal-to-noise ratios.

Other reporting systems may also be useful in the cloning strategy, such as an immunofluorescence/ immunocytochemical approach in COS-1 cells that also relies on gene induction. Commercially available anti-GFP Abs (Clontech) or anti-β-galactosidase Abs (Promega Biotech, Inc.) can be used to identify transfected COS-1 cells in which ectopic gene expression has been induced. Or, a plasmid can be constructed in which CRE or AP-1 drives expression of a cell-surface protein to which Abs are available, such as nerve growth factor receptor (Johnson et al. 1986), and FACS sorting can be used to identify positive cells. Alternatively, a rapid effect of activation of the ThrR/hCTR in COS-1 cells can be monitored. Stimulation of an acute elevation in cytoplasmic Ca^{2+} using Fluo-3 (Molecular Probes) and fluorescence microscopy can be measured. Fluorescent calcium indicators (Geras Raaka and Gershengorn 1987) can be used.

The strategy in accordance with the subject invention for the design of the library suits the purpose for which it is intended to be used because: i) It removes the putative EC N-terminal domain of hCTR to which intact, native CT binds with high affinity. Without hCTR's N-terminus, the possibility of finding a peptide that acts indirectly through hCTR's N- terminus is eliminated. ii) It produces receptors that activate

only upon addition of thrombin. This allows for receptors to be active only during the experimental period, avoiding the cellular counterregulatory mechanisms associated with prolonged stimulation (desensitization, down regulation), which can attenuate the detecting signal. iii) A tethered agonist increases the local effective concentration of the ligand enormously. This reduces the possibility that peptide antagonists, if present in the same pool of an untethered peptide library, could interfere with the detection of peptide agonists.

EXAMPLE III

Peptide Negative Antagonists of a GPCR of HHV-8

This example relates to a newly described GPCR that is encoded in the genome of human herpesvirus-8 (HHV-8) (or Kaposi's sarcoma-associated herpesvirus - KSHV) (Cesarman et al. 1996), which is a virus that was first identified in Kaposi's sarcoma (KS) tissues from patients with AIDS and has now been found in KS tissues from human immunodeficiency virus (HIV)-negative patients, in tissues from patients with Castleman's disease and in some B-cell lymphomas (Chang et al. 1994). This receptor is referred to as HHV8 GPCR. The objective of this example is to identify peptides that are high affinity negative antagonists of this constitutively active HHV8 GPCR. A constitutively active receptor is a receptor that exhibits agonist-independent signalling activity (Lefkowitz et al. 1993). Negative antagonists (or inverse agonists) are compounds that are capable of inhibiting the signalling activity of a constitutively active receptor (Schutz and Freissmuth 1992). Neutral antagonists inhibit the action of agonists but do not affect agonist-independent activity. Neutral antagonists would inhibit signalling by HHV8 GPCR when it is activated by natural or synthetic agonists, for

example, interleukin-8 (IL-8) that has been shown to activate HHV8 GPCR in *Xenopus laevis* oocytes, whereas negative antagonists would inhibit "basal" signalling by HHV8 GPCR.

The HHV8 GPCR is a protein of 342 amino acids that has the features of a GPCR including seven hydrophobic, putative transmembrane-spanning domains (Cesarman et al. 1996). Fig. 7 illustrates the putative two-dimensional topology of HHV8 GPCR in the cell-surface membrane. The *top* of the diagram represents the extracellular space (E), the *middle* portion represents the transmembrane (TM) domain and the *bottom* portion represents the intracellular space (C). Each circle represents an amino acid residue designated by the single letter code. The HHV8 GPCR is a receptor that signals via the phosphoinositide-inositol 1,4,5-trisphosphate-calcium cascade (Berridge 1993).

Discovery of peptide negative antagonists of HHV8 GPCR

It is now appreciated that receptors can attain an active conformation in the absence of agonist and manifest constitutive, that is, agonist-independent activity (Lefkowitz et al. 1993). This has led to renewed acceptance of the concept that receptors can change conformation spontaneously and oscillate between active and inactive states (for review, see Leff 1995). Some drugs, termed negative antagonists or inverse agonists, appear capable of constraining receptors in an inactive state (Samama et al. 1994). Negative antagonism is demonstrated when a drug binds to a receptor that exhibits constitutive activity and reduces this activity. It is important to discover agents that exhibit negative antagonistic properties toward HHV8 GPCR to use in exploring the role of HHV8 GPCR during HHV-8 infection in studies in cells in tissue culture and in intact animals.

The subject invention provides a strategy for discovery of small peptide negative antagonists of HHV8 GPCR. A tethered, combinatorial library is used to clone pentapeptides that are negative antagonists of HHV8 GPCR. A pentapeptide library is chosen based on the fact small peptides are effective negative antagonists and the number of clones is workable. The library contains all 20 natural amino acids at each of the five positions and therefore has a complexity of $20^5 = 3.2 \times 10^6$ possible combinations. This approach is chosen because although there is a good deal of information available regarding IL-8 binding (see above), little is known regarding the specific interactions between IL-8 and IL-8Rs that cause activation (Leong et al. 1994). In fact, this is true for GPCRs in general (Van Rhee and Jacobson 1996). Moreover, there is even less known about specific interactions that may inactivate a constitutively active receptor (Schutz and Freissmuth 1992). Thus, insufficient information is available to "rationally" design small peptides with negative antagonist activities. Thus, discovery of negative antagonist peptides for HHV8 GPCR may best be accomplished by using combinatorial peptide libraries. With this approach, 3.2 million random peptides of five amino acids in length are tested for activity and those that inactivate HHV8 GPCRs are identified by sib selection.

Discovery of high affinity, specific pentapeptide negative antagonists of HHV8 GPCR

To discover small peptides that can serve as negative antagonists (or inverse agonists) for HHV8 GPCR, a combinatorial peptide library is constructed that expresses random pentapeptides tethered to the seven TM helical bundle of HHV8 GPCR. This strategy is based on the conclusion that one (or several) pentapeptides will interact with the TM bundle or

extracellular loops, or both of HHV8 GPCR in a manner similar to that by which other small peptide antagonists interact with other GPCRs, such as receptors for opioid peptides (Costa and Herz 1989; Costa et al. 1992) and bradykinin (Leeb-Lundberg et al. 1994), and by a similar mechanism inactivate HHV8 GPCR.

A pentapeptide library is chosen based on the fact that peptides of this size have been shown to be negative antagonists of other GPCRs and the resulting number of clones is workable. The library contains all 20 natural amino acids at each of the five positions and, therefore, has a complexity of $20^5 = 3.2 \times 10^6$ possible compounds. The library is constructed by taking the cDNA sequence of HHV8 GPCR and substituting the sequence that normally encodes HHV8 GPCR's N-terminal extracellular domain by a DNA sequence that encodes the N-terminal ectodomain of the thrombin receptor (ThrR) from just after the activating peptide to the beginning of TM-1; that is, the sequence of the native ThrR from its N-terminus up to and including its activating peptide, Ser-Phe-Leu-Leu-Arg-Asn (SEQ ID NO:43:SFLLRN), is deleted. The chimeric ThrR/HHV8 GPCR primary amino acid sequence begins at its N-terminus with the variable pentapeptide sequence ("library"), which is substituting for SFLLRN, followed by the ThrR amino terminal sequence distal to the SFLLRN sequence (from immediately after SFLLRN to the beginning of TM-1) followed by the HHV8GPCR sequence from the beginning of TM-1 to the carboxyl end (Fig. 8). The distal N-terminal sequence of the ThrR is chosen rather than that of HHV8 GPCR because this sequence allows the pentapeptide library sequences on each ThrR/HHV8 GPCR chimera to be directed into the remainder of the receptor as the exposed N-terminal peptide of ThrR is guided into the receptor's "body". The major difference is that the pentapeptide library is the N-terminus of the ThrR/HHV8 GPCR tethered to the

remainder of the receptor, that is, in a position that in the native ThrR allows it to serve as an agonist but allows it in the chimeric receptor to serve as a negative antagonist. No cleavage is necessary to expose the N-terminus pentapeptide sequence. Therefore, the N-terminus of expressed receptors are random pentapeptides that can act as negative antagonists with regard to the constitutive activity of HHV8 GPCRs as soon as the chimeric receptor is expressed. The library is constructed without the need to cleave off a "blocking" sequence in order to expose the pentapeptide because it is desirable for the pentapeptide to inactivate the chimeric receptor as soon as it is expressed on the cell surface. Thus, monitoring is for inactivation of a "basal" signalling activity of the chimeric ThrR/HHV8 GPCR.

Fig. 8 shows the putative topology of the chimera ThrR/HHV8 GPCR as it is predicted to be in the cell surface membrane of transfected COS-1 cells. The *top* of the diagram represents the extracellular space (E), the *middle* portion represents the transmembrane (TM) domain and the *bottom* portion represents the intracellular space (C). The first five filled circles represent individual amino acids that are part of the pentapeptide library; each filled circle represents 20 amino acids. The seventy unfilled circles represent the individual amino acid residues of the native ThrR sequence from just after the activating peptide (SFLLRN) to the beginning of TM-1. Each circle with a letter in it represents an amino acid residue designated by the single letter code of HHV8 GPCR.

To monitor for cell surface expression of the chimeric receptors, antibodies to the extracellular domain of HHV8 GPCR are used, specifically antibodies to the large extracellular loop 2.

The cDNA sequence encoding the new N-terminus of the chimeric ThrR/HHV8 GPCR, consisting of a

prolactin leader (or signal) peptide, which is cleaved after directing the protein to the cell surface membrane, followed by the pentapeptide library and the distal sequence of the N-terminus of ThrR is constructed by gene synthesis. It consists of a DNA segment of approximately 210 base pairs encoding 70 amino acids that are ligated in frame through an appropriate restriction endonuclease cleavage site that is created in the HHV8 GPCR cDNA at a position encoding the amino acids that constitute the transition between the N-terminus and the first TM domain. After ligation into a mammalian expression vector, *Escherichia coli* is transformed by electroporation and the transformants are subdivided into pools whose maximal workable complexity is determined according to the efficiency of mammalian cell transfection and/or sensitivity of the detection system(s).

The success of expression cloning strategies, such as the one of the subject invention, is dependent on the reporter (or detection) system. An amplified reporter system is used in accordance with the subject invention which is based on the second messenger system triggered by HHV8 GPCR. HHV8 GPCR is a GPCR that in COS-1 cells appears to couple through a G protein to the enzyme phospholipase C causing generation of the second messengers inositol 1,4,5-trisphosphate (IP_3), which causes a rise in intracellular free Ca^{2+}, and 1,2-diacylglycerol, which activates protein kinase C (Nussenzveig et al. 1994). Activated protein kinase C triggers gene induction through specific motifs using transcription factors, such as the *fos-jun*-AP-1 system (Deutsch et al. 1990; Schadlow et al. 1992). This reporter system works in COS-1 cells since constitutive activity of HHV8 GPCR is detected using a reporter plasmid containing a minimal promoter of the human *c-fos* gene into which a AP-1 binding motif is engineered driving transcription of the gene for the

enzyme luciferase (pAP-1/LUC), whose activity is detected by a chemiluminescent reaction. Unfortunately, the use of the enzyme activity of the luciferase reporter system requires the preparation of cell extracts and, therefore, monitors induction in a population of cells. To be able to identify receptors that are turned off by negative antagonistic activity of the tethered pentapeptide, a single "hit" in a very large number of negatives needs to be measured. Therefore, a single cell assay is needed. For the reporter, luciferase is replaced with β-galactosidase that can be readily measured in individual cells.

A two-reporter system was devised for discovery of negative antagonists that use gene induction in COS-1 cells. β-galactosidase is used as a reporter enzyme in transfected COS-1 cells. This assay takes advantage of the amplification of the enzyme activity of the reporter, with an easily determined color reaction as endpoint, and of the over-expression of receptors with tethered negative antagonists in COS-1 cells because of replication of the plasmids introduced. These experiments are performed by co-transfecting portions of the plasmid library and a plasmid encoding AP-1/β-galactosidase constructs into COS-1 cells so as to amplify expression by plasmid replication using the simian virus-40 origin of replication in the vector. This enhances the signal/noise ratio substantially. The signal is further increased because the construct used has a nuclear localization signal ligated to the β-galactosidase that allows the protein to concentrate in the nucleus (Hersh et al. 1995). The construct containing β-galactosidase with a nuclear localization signal was shown to express in the nucleus of transfected COS-1 cells. Single clones that exhibit negative antagonistic activity, as measured by decreased color reaction, are isolated using sib

selection, which consists of successive subdivision and amplification of positive pools of clones. The optimal time after transfection to assay β-galactosidase activity is determined empirically as this involves a prolonged response on gene induction and the kinetics of this response vary with different activators (receptors) and in different cells.

A second reporter gene is used to identify cells that have been transfected and are expressing foreign proteins to distinguish them from cells that have not been transfected. This is a crucial distinction for this approach because differentiation between cells that have the capacity to express the specific reporter gene but are not (or in which expression has been diminished) because transcription has been inhibited, from cells that are not expressing the reporter gene because they are not transfected, is necessary. Because the β-galactosidase activity is expressed in the nucleus, it has a different localization than the nonspecific reporter of transfection. The nonspecific reporter of transfection is a construct containing a mutant of the human placental alkaline phosphatase gene (Tate et al. 1990) that is targeted to the cytoplasm under the control of a cytomegalovirus promoter; this promoter is not affected by HHV8 GPCR and is active in all transfected cells. Thus, one can monitor for 3 types of cells: 1) cells in which β-galactosidase is expressed at high levels in the nucleus and alkaline phosphatase is expressed in the cytoplasm - these are transfected cells that do not express receptors that contain a peptide that has negative antagonistic activity because expression of β-galactosidase is induced by the constitutive signalling activity of HHV8 GPCR; 2) cells in which β-galactosidase is not expressed in the nucleus and alkaline phosphatase is not expressed in the cytoplasm - these are cells that have not been

transfected; and 3) cells in which β-galactosidase is not expressed (or is expressed at low levels in the nucleus) and alkaline phosphatase is expressed in the cytoplasm - these are transfected cells that express receptors that contain a peptide that has negative antagonistic activity.

Other reporting systems may also be useful in the cloning strategy, such as the yeast bioassay system discussed above or an immunofluorescence/ immunocytochemical approach in COS-1 cells that also relies on gene induction. Commercially available anti-β-galactosidase antibodies (Promega Biotech, Inc.) can be used to identify transfected COS-1 cells in which ectopic gene expression has been modulated. Or, a plasmid can be constructed in which AP-1 drives the expression of a cell-surface protein to which Abs are available, such as the nerve growth factor receptor (Johnson et al. 1986).

The strategy devised in the design of the library suits the purpose for which it is intended to be used, because a tethered negative antagonist increases the local effective concentration of the ligand enormously. This also reduces the possibility that neutral antagonists or agonists, if present in the same pool of an untethered peptide library, could interfere with the detection of peptide negative antagonists.

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those of ordinary skill in the relevant art that various modifications, additions, substitutions and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Cornell Research Foundation, Inc.

(ii) TITLE OF INVENTION: STRATEGY TO CLONE DRUGS FOR G PROTEIN COUPLED RECEPTORS

(iii) NUMBER OF SEQUENCES: 44

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 63 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Leu Asp Ala Thr Leu Asp Pro Arg Ser Phe Leu Leu Arg Asn Pro Asn
1 5 10 15

Asp Lys Tyr Glu Pro Phe Trp Glu Asp Glu Glu Lys Asn Glu Ser Gly
20 25 30

Leu Thr Glu Tyr Arg Leu Val Ser Ile Asn Lys Ser Ser Pro Leu Gln
35 40 45

Lys Gln Leu Pro Ala Phe Ile Ser Glu Asp Ala Ser Gly Tyr Leu
50 55 60

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 436 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Asp Ser Lys Gly Ser Ser Gln Lys Gly Ser Arg Leu Leu Leu Leu
1 5 10 15

Leu Val Val Ser Asn Leu Leu Leu Cys Gln Gly Val Val Ser Asp Tyr
20 25 30

Lys Asp Asp Asp Asp Lys Leu Asp Ala Thr Leu Asp Pro Arg Xaa Xaa
35 40 45

Xaa Xaa Xaa Asn Pro Asn Asp Lys Tyr Glu Pro Phe Trp Glu Asp Glu
50 55 60

Glu Lys Asn Glu Ser Gly Leu Thr Glu Tyr Arg Leu Val Ser Ile Asn
65 70 75 80

Lys Ser Ser Pro Leu Gln Lys Gln Leu Pro Ala Phe Ile Ser Glu Asp
85 90 95

Ala Ser Gly Tyr Leu Gly Tyr Asn Ile Leu Arg Val Leu Ile Trp Phe
100 105 110

Ile Ser Ile Leu Ala Ile Thr Gly Asn Ile Ile Val Leu Val Ile Leu
115 120 125

Thr Thr Ser Gln Tyr Lys Leu Thr Val Pro Arg Phe Leu Met Cys Asn
130 135 140

Leu Ala Phe Ala Asp Leu Cys Ile Gly Ile Tyr Leu Leu Leu Ile Ala
145 150 155 160

Ser Val Asp Ile His Thr Lys Ser Gln Tyr His Asn Tyr Ala Ile Asp
165 170 175

Trp Gln Thr Gly Ala Gly Cys Asp Ala Ala Gly Phe Phe Thr Val Phe
180 185 190

Ala Ser Glu Leu Ser Val Tyr Thr Leu Thr Ala Ile Thr Leu Glu Arg
195 200 205

Trp His Thr Ile Thr His Ala Met Gln Leu Asp Cys Lys Val Gln Leu
210 215 220

Arg His Ala Ala Ser Val Met Val Met Gly Trp Ile Phe Ala Phe Ala
225 230 235 240

Ala Ala Leu Phe Pro Ile Phe Gly Ile Ser Ser Tyr Met Lys Val Ser
245 250 255

Ile Cys Leu Pro Met Asp Ile Asp Ser Pro Leu Ser Gln Leu Tyr Val
260 265 270

Met Ser Leu Leu Val Leu Asn Val Leu Ala Phe Val Val Ile Cys Gly
275 280 285

Cys Tyr Ile His Ile Tyr Leu Thr Val Arg Asn Pro Asn Ile Val Ser
290 295 300

Ser Ser Ser Asp Thr Arg Ile Ala Lys Arg Met Ala Met Leu Ile Phe
305 310 315 320

Thr Asp Phe Leu Cys Met Ala Pro Ile Ser Phe Phe Ala Ile Ser Ala
325 330 335

Ser Leu Lys Val Pro Leu Ile Thr Val Ser Lys Ala Lys Ile Leu Leu
340 345 350

Val Leu Phe His Pro Ile Asn Ser Cys Ala Asn Pro Phe Leu Tyr Ala
355 360 365

Ile Phe Thr Lys Asn Phe Arg Arg Asp Phe Phe Ile Leu Leu Ser Lys
370 375 380

Cys Gly Cys Tyr Glu Met Gln Ala Gln Ile Tyr Arg Thr Glu Thr Ser
385 390 395 400

Ser Thr Val His Asn Thr His Pro Arg Asn Gly His Cys Ser Ser Ala
405 410 415

Pro Arg Val Thr Asn Gly Ser Thr Tyr Ile Leu Val Pro Leu Ser His
420 425 430

Leu Ala Gln Asn
435

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Asp Ser Lys Gly Ser Ser Gln Lys Gly Ser Arg Leu Leu Leu Leu
1   5   10   15

Leu Val Val Ser Asn Leu Leu Leu Cys Gln Gly Val Val Ser
            20   25   30

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Asp Tyr Lys Asp Asp Asp Asp Lys
1   5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Xaa Xaa Xaa Xaa Xaa
1   5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AATTCCACCA TGGACTCCAA GGGCTCGAGC CAGAAGGGAT CTAGACTGCT 50

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CAGCAGCAGT CTAGATCCCT TCTGGCTCGA GCCCTTGGAG TCCATGGTGG 50

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCTGCTGCTG GTGGTGAGCA ACCTGCTGCT GTGCCAGGGC GTCGTG 46

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGCTCACGAC GCCCTGGCAC AGCAGCAGGT TGCTCACCAC CAGCAG 46

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AGCGACTACA AGGACGACGA CGACAAGCTT CCTGCCTTTT 40

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CGAAAAGGCA GGAAGCTTGT CGTCGTCGTC CTTGTAGT 38

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TATGCCACCT TTTGGGAGGA TGAGGAGAAA AATGAAAGTG GGTTAACTGA ATAC 54

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGAAGAGGAC TGCTTTTATT GATGGAGACT AATCTGTATT CAGTTAACCC ACTTTC 56

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CAATAAAAGC AGTCCTCTTC AAAAACAACT TCCTGCATTC ATCTCAGAAG ATGCC 55

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GTCAGGTAAC CGGAGGCATC TTCTGAGATG AATGCAAG 38

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TGAAGGTTAC CTGGGGTACA ACATCCTCAG AGTCC 35

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TCACGCGGCC GCTTAGTTTT GGGCTAAATG ACTTAGAGG 39

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AATTCGGCTT 10

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AGCCG 5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CAGTCGATCG CATAGTTGTG ATATTGGCTC 30

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AACAGCTATG ACCATG 16

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CCATCCGCGG AATGGCCACT GCTCTTCAGC 30

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GTAAAACGAC GGCCAGT 17

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TATGCGATCG ACTGGCAAAC TGGGGCAGG 29

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CATTCCGCGG ATGGGTGTTG TGGACAGTG 29

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

AGCTTGATGC CACGCTATGG CCCTAGGTAA GTGATATGCC ACCTT 45

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GTGGCATATC ACTTACCTAG GGCCATAGCG TGGCATCA 38

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

AGATCCCCGG AACCCCAATG ATAAATATGA ACCCTT 36

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CCGGGGATCT AGC 13

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GGTTCATATT TATC 14

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1300 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

AATTCCACCA TGGACTCCAA GGGCTCGAGC CAGAAGGGAT CTAGACTGCT GCTGCTGCTG 60
GTGGTGAGCA ACCTGCTGCT GTGCCAGGGC GTCGTGAGCG ACTACAAGGA CGACGACGAC 120
AAGCTTGATG CCACGCTATG GCCCTAGGTA AGTGATATGC CACCTTTTGG GAGGATGAGG 180
AGAAAAATGA AAGTGGGTTA ACTGAATACA GATTAGTCTC CATCAATAAA AGCAGTCCTC 240
TTCAAAAACA ACTTCCTGCA TTCATCTCAG AAGATGCCTC CGGTTACCTG GGGTACAACA 300
TCCTCAGAGT CCTGATATGG TTTATCAGCA TCCTGGCCAT CACTGGGAAC ATCATAGTGC 360
TAGTGATCCT AACTACCAGC CAATATAAAC TCACAGTCCC CAGGTTCCTT ATGTGCAACC 420
TGGCCTTTGC TGATCTCTGC ATTGGAATCT ACCTGCTGCT CATTGCATCA GTTGATATCC 480
ATACCAAGAG CCAATATCAC AACTATGCGA TCGACTGGCA AACTGGGGCA GGCTGTGATG 540
CTGCTGGCTT TTTCACTGTC TTTGCCAGTG AGCTGTCAGT CTACACTCTG ACAGCTATCA 600
CCTTGGAAAG ATGGCATACC ATCACGCATG CCATGCAGCT GGACTGCAAG GTGCAGCTCC 660
GCCATGCTGC CAGTGTCATG GTGATGGGCT GGATTTTTGC TTTTGCAGCT GCCCTCTTTC 720
CCATCTTTGG CATCAGCAGC TACATGAAGG TGAGCATCTG CCTGCCCATG GATATTGACA 780
GCCCTTTGTC ACAGCTGTAT GTCATGTCCC TCCTTGTGCT CAATGTCCTG GCCTTTGTGG 840
TCATCTGTGG CTGCTATATC CACATCTACC TCACAGTGCG GAACCCCAAC ATCGTGTCCT 900
CCTCTAGTGA CACCAGGATC GCCAAGCGCA TGGCCATGCT CATCTTCACT GACTTCCTCT 960
GCATGGCACC CATTTCTTTC TTTGCCATTT CTGCCTCCCT CAAGGTGCCC CTCATCACTG 1020
TGTCCAAAGC AAAGATTCTG CTGGTTCTGT TTCACCCCAT CAACTCCTGT GCCAACCCCT 1080
TCCTCTATGC CATCTTTACC AAAAACTTTC GCAGAGATTT CTTCATTCTG CTGAGCAAGT 1140
GTGGCTGCTA TGAAATGCAA GCCCAAATTT ATAGGACAGA AACTTCATCC ACTGTCCACA 1200
ACACCCATCC GCGGAATGGC CACTGCTCTT CAGCTCCCAG AGTCACCAAT GGTTCCACTT 1260
ACATACTTGT CCCTCTAAGT CATTTAGCCC AAAACTAAGC 1300

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1298 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

```
GGCCGCTTAG TTTTGGGCTA AATGACTTAG AGGGACAAGT ATGTAAGTGG AACCATTGGT      60
GACTCTGGGA GCTGAAGAGC AGTGGCCATT CCGCGGATGG GTGTTGTGGA CAGTGGATGA     120
AGTTTCTGTC CTATAAATTT GGGCTTGCAT TTCATAGCAG CCACACTTGC TCAGCAGAAT     180
GAAGAAATCT CTGCGAAAGT TTTTGGTAAA GATGGCATAG AGGAAGGGGT TGGCACAGGA     240
GTTGATGGGG TGAAACAGAA CCAGCAGAAT CTTTGCTTTG GACACAGTGA TGAGGGGCAC     300
CTTGAGGGAG GCAGAAATGG CAAAGAAAGA AATGGGTGCC ATGCAGAGGA AGTCAGTGAA     360
GATGAGCATG GCCATGCGCT TGGCGATCCT GGTGTCACTA GAGGAGGACA CGATGTTGGG     420
GTTCCGCACT GTGAGGTAGA TGTGGATATA GCAGCCACAG ATGACCACAA AGGCCAGGAC     480
ATTGAGCACA AGGAGGGACA TGACATACAG CTGTGACAAA GGGCTGTCAA TATCCATGGG     540
CAGGCAGATG CTCACCTTCA TGTAGCTGCT GATGCCAAAG ATGGGAAAGA GGGCAGCTGC     600
AAAAGCAAAA ATCCAGCCCA TCACCATGAC ACTGGCAGCA TGGCGGAGCT GCACCTTGCA     660
GTCCAGCTGC ATGGCATGCG TGATGGTATG CCATCTTTCC AAGGTGATAG CTGTCAGAGT     720
GTAGACTGAC AGCTCACTGG CAAAGACAGT GAAAAAGCCA GCAGCATCAC AGCCTGCCCC     780
AGTTTGCCAG TCGATCGCAT AGTTGTGATA TTGGCTCTTG GTATGGATAT CAACTGATGC     840
AATGAGCAGC AGGTAGATTC CAATGCAGAG ATCAGCAAAG GCCAGGTTGC ACATAAGGAA     900
CCTGGGGACT GTGAGTTTAT ATTGGCTGGT AGTTAGGATC ACTAGCACTA TGATGTTCCC     960
AGTGATGGCC AGGATGCTGA TAAACCATAT CAGCACTCTG AGGATGTTGT ACCCCAGGTA    1020
ACCGGAGGCA TCTTCTGAGA TGAATGCAGG AAGTTGTTTT TGAAGAGGAC TGCTTTTATT    1080
GATGGAGACT AAACTGTATT CAGTTAACCC ACTTTCATTT TTCTCCTCAT CCTCCCAAAA    1140
GGGCATATCA CTTACCTAGG GCCATAGCGT GGCATCAAGC TTGTCGTCGT CGTCCTTGTA    1200
GTCGCTCACG ACGCCCTGGC ACAGCAGCAG GTTGCTCACC ACCAGCAGCA GCAGCAGTCT    1260
AGATCCCTTC TGGCTCGAGC CCTTGGAGTC CATGGTGG                            1298
```

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 420 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

```
Met Asp Ser Lys Gly Ser Ser Gln Lys Gly Ser Arg Leu Leu Leu Leu
1               5                   10                  15

Leu Val Val Ser Asn Leu Leu Leu Cys Gln Gly Val Val Ser Asp Tyr
            20                  25                  30

Lys Asp Asp Asp Asp Lys Leu Asp Ala Thr Leu Phe Trp Glu Asp Glu
        35                  40                  45

Glu Lys Asn Glu Ser Gly Leu Thr Glu Tyr Arg Leu Val Ser Ile Asn
    50                  55                  60

Lys Ser Ser Pro Leu Gln Lys Gln Leu Pro Ala Phe Ile Ser Glu Asp
65                  70                  75                  80

Ala Ser Gly Tyr Leu Gly Tyr Asn Ile Leu Arg Val Leu Ile Trp Phe
                85                  90                  95

Ile Ser Ile Leu Ala Ile Thr Gly Asn Ile Ile Val Leu Val Ile Leu
            100                 105                 110

Thr Thr Ser Gln Tyr Lys Leu Thr Val Pro Arg Phe Leu Met Cys Asn
        115                 120                 125

Leu Ala Phe Ala Asp Leu Cys Ile Gly Ile Tyr Leu Leu Leu Ile Ala
    130                 135                 140

Ser Val Asp Ile His Thr Lys Ser Gln Tyr His Asn Tyr Ala Ile Asp
145                 150                 155                 160

Trp Gln Thr Gly Ala Gly Cys Asp Ala Ala Gly Phe Phe Thr Val Phe
                165                 170                 175

Ala Ser Glu Leu Ser Val Tyr Thr Leu Thr Ala Ile Thr Leu Glu Arg
            180                 185                 190

Trp His Thr Ile Thr His Ala Met Gln Leu Asp Cys Lys Val Gln Leu
        195                 200                 205

Arg His Ala Ala Ser Val Met Val Met Gly Trp Ile Phe Ala Phe Ala
    210                 215                 220

Ala Ala Leu Phe Pro Ile Phe Gly Ile Ser Ser Tyr Met Lys Val Ser
225                 230                 235                 240

Ile Cys Leu Pro Met Asp Ile Asp Ser Pro Leu Ser Gln Leu Tyr Val
                245                 250                 255
```

Met Ser Leu Leu Val Leu Asn Val Leu Ala Phe Val Val Ile Cys Gly
260 265 270

Cys Tyr Ile His Ile Tyr Leu Thr Val Arg Asn Pro Asn Ile Val Ser
275 280 285

Ser Ser Ser Asp Thr Arg Ile Ala Lys Arg Met Ala Met Leu Ile Phe
290 295 300

Thr Asp Phe Leu Cys Met Ala Pro Ile Ser Phe Phe Ala Ile Ser Ala
305 310 315 320

Ser Leu Lys Val Pro Leu Ile Thr Val Ser Lys Ala Lys Ile Leu Leu
325 330 335

Val Leu Phe His Pro Ile Asn Ser Cys Ala Asn Pro Phe Leu Tyr Ala
340 345 350

Ile Phe Thr Lys Asn Phe Arg Arg Asp Phe Phe Ile Leu Leu Ser Lys
355 360 365

Cys Gly Cys Tyr Glu Met Gln Ala Gln Ile Tyr Arg Thr Glu Thr Ser
370 375 380

Ser Thr Val His Asn Thr His Pro Arg Asn Gly His Cys Ser Ser Ala
385 390 395 400

Pro Arg Val Thr Asn Gly Ser Thr Tyr Ile Leu Val Pro Leu Ser His
405 410 415

Leu Ala Gln Asn
420

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 85 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Met Asp Ser Lys Gly Ser Ser Gln Lys Gly Ser Arg Leu Leu Leu Leu
1 5 10 15

Leu Val Val Ser Asn Leu Leu Leu Cys Gln Gly Val Val Ser Xaa Xaa
20 25 30

Xaa Xaa Xaa Asn Pro Asn Asp Lys Tyr Glu Pro Phe Trp Glu Asp Glu
35 40 45

Glu Lys Asn Glu Ser Gly Leu Thr Glu Tyr Arg Leu Val Ser Ile Asn
50 55 60

Lys Ser Ser Pro Leu Gln Lys Gln Leu Pro Ala Phe Ile Ser Glu Asp
65 70 75 80

Ala Ser Gly Tyr Leu
85

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

ATCAAGCTTG TCGTCGTCGT CCTTGTAGTC GCTCACCACG CCCTG 45

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1300 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

AATTCCACCA TGGACTCCAA GGGCTCGAGC CAGAAGGGAT CTAGACTGCT GCTGCTGCTG 60
GTGGTGAGCA ACCTGCTGCT GTGCCAGGGC GTGGTGAGCG ACTACAAGGA CGACGACGAC 120
AAGCTTGATG CCACGCTATG GCCCTAGGTA AGTGATATGC CACCTTTTGG GAGGATGAGG 180
AGAAAAATGA AAGTGGGTTA ACTGAATACA GATTAGTCTC CATCAATAAA AGCAGTCCTC 240
TTCAAAAACA ACTTCCTGCA TTCATCTCAG AAGATGCCTC CGGTTACCTG GGGTACAACA 300
TCCTCAGAGT CCTGATATGG TTTATCAGCA TCCTGGCCAT CACTGGGAAC ATCATAGTGC 360
TAGTGATCCT AACTACCAGC CAATATAAAC TCACAGTCCC CAGGTTCCTT ATGTGCAACC 420
TGGCCTTTGC TGATCTCTGC ATTGGAATCT ACCTGCTGCT CATTGCATCA GTTGATATCC 480
ATACCAAGAG CCAATATCAC AACTATGCGA TCGACTGGCA AACTGGGGCA GGCTGTGATG 540
CTGCTGGCTT TTTCACTGTC TTTGCCAGTG AGCTGTCAGT CTACACTCTG ACAGCTATCA 600
CCTTGGAAAG ATGGCATACC ATCACGCATG CCATGCAGCT GGACTGCAAG GTGCAGCTCC 660
GCCATGCTGC CAGTGTCATG GTGATGGGCT GGATTTTTGC TTTTGCAGCT GCCCTCTTTC 720
CCATCTTTGG CATCAGCAGC TACATGAAGG TGAGCATCTG CCTGCCCATG GATATTGACA 780

GCCCTTTGTC ACAGCTGTAT GTCATGTCCC TCCTTGTGCT CAATGTCCTG GCCTTTGTGG 840

TCATCTGTGG CTGCTATATC CACATCTACC TCACAGTGCG GAACCCCAAC ATCGTGTCCT 900

CCTCTAGTGA CACCAGGATC GCCAAGCGCA TGGCCATGCT CATCTTCACT GACTTCCTCT 960

GCATGGCACC CATTTCTTTC TTTGCCATTT CTGCCTCCCT CAAGGTGCCC CTCATCACTG 1020

TGTCCAAAGC AAAGATTCTG CTGGTTCTGT TTCACCCCAT CAACTCCTGT GCCAACCCCT 1080

TCCTCTATGC CATCTTTACC AAAAACTTTC GCAGAGATTT CTTCATTCTG CTGAGCAAGT 1140

GTGGCTGCTA TGAAATGCAA GCCCAAATTT ATAGGACAGA AACTTCATCC ACTGTCCACA 1200

ACACCCATCC GCGGAATGGC CACTGCTCTT CAGCTCCCAG AGTCACCAAT GGTTCCACTT 1260

ACATACTTGT CCCTCTAAGT CATTTAGCCC AAAACTAAGC 1300

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1300 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GGCCGCTTAG TTTTGGGCTA AATGACTTAG AGGGACAAGT ATGTAAGTGG AACCATTGGT 60

GACTCTGGGA GCTGAAGAGC AGTGGCCATT CCGCGGATGG GTGTTGTGGA CAGTGGATGA 120

AGTTTCTGTC CTATAAATTT GGGCTTGCAT TTCATAGCAG CCACACTTGC TCAGCAGAAT 180

GAAGAAATCT CTGCGAAAGT TTTTGGTAAA GATGGCATAG AGGAAGGGGT TGGCACAGGA 240

GTTGATGGGG TGAAACAGAA CCAGCAGAAT CTTTGCTTTG GACACAGTGA TGAGGGGCAC 300

CTTGAGGGAG GCAGAAATGG CAAAGAAAGA AATGGGTGCC ATGCAGAGGA AGTCAGTGAA 360

GATGAGCATG GCCATGCGCT TGGCGATCCT GGTGTCACTA GAGGAGGACA CGATGTTGGG 420

GTTCCGCACT GTGAGGTAGA TGTGGATATA GCAGCCACAG ATGACCACAA AGGCCAGGAC 480

ATTGAGCACA AGGAGGGACA TGACATACAG CTGTGACAAA GGGCTGTCAA TATCCATGGG 540

CAGGCAGATG CTCACCTTCA TGTAGCTGCT GATGCCAAAG ATGGGAAAGA GGGCAGCTGC 600

AAAAGCAAAA ATCCAGCCCA TCACCATGAC ACTGGCAGCA TGGCGGAGCT GCACCTTGCA 660

GTCCAGCTGC ATGGCATGCG TGATGGTATG CCATCTTTCC AAGGTGATAG CTGTCAGAGT 720

GTAGACTGAC AGCTCACTGG CAAAGACAGT GAAAAAGCCA GCAGCATCAC AGCCTGCCCC 780

AGTTTGCCAG TCGATCGCAT AGTTGTGATA TTCGCTCTTG GTATGGATAT CAACTGATGC 840

AATGAGCAGC AGGTAGATTC CAATGCAGAG ATCAGCAAAG GCCAGGTTGC ACATAAGGAA 900

CCTGGGGACT GTGAGTTTAT ATTGGCTGGT AGTTAGGATC ACTAGCACTA TGATGTTCCC 960

AGTGATGGCC AGGATGCTGA TAAACCATAT CAGGACTCTG AGGATGTTGT ACCCCAGGTA 1020

ACCGGAGGCA TCTTCTGAGA TGAATGCAGG AAGTTGTTTT TGAAGAGGAC TGCTTTTATT 1080

GATGGAGACT AAACTGTATT CAGTTAACCC ACTTTCATTT TTCTCCTCAT CCTCCCAAAA 1140

GGTGGCATAT CACTTACCTA GGGCCATAGC GTGGCATCAA GCTTGTCGTC GTCGTCCTTG 1200

TAGTCGCTCA CCACGCCCTG GCACAGCAGC AGGTTGCTCA CCACCAGCAG CAGCAGCAGT 1260

CTAGATCCCT TCTGGCTCGA GCCCTTGGAG TCCATGGTGG 1300

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 423 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Met Asp Ser Lys Gly Ser Ser Gln Lys Gly Ser Arg Leu Leu Leu Leu
1 5 10 15

Leu Val Val Ser Asn Leu Leu Leu Cys Gln Gly Val Val Ser Asp Tyr
20 25 30

Lys Asp Asp Asp Asp Lys Leu Asp Ala Thr Leu Leu Trp Pro Phe Trp
35 40 45

Glu Asp Glu Glu Lys Asn Glu Ser Gly Leu Thr Glu Tyr Arg Leu Val
50 55 60

Ser Ile Asn Lys Ser Ser Pro Leu Gln Lys Gln Leu Pro Ala Phe Ile
65 70 75 80

Ser Glu Asp Ala Ser Gly Tyr Leu Gly Tyr Asn Ile Leu Arg Val Leu
85 90 95

Ile Trp Phe Ile Ser Ile Leu Ala Ile Thr Gly Asn Ile Ile Val Leu
100 105 110

Val Ile Leu Thr Thr Ser Gln Tyr Lys Leu Thr Val Pro Arg Phe Leu
115 120 125

Met Cys Asn Leu Ala Phe Ala Asp Leu Cys Ile Gly Ile Tyr Leu Leu
130 135 140

Leu Ile Ala Ser Val Asp Ile His Thr Lys Ser Gln Tyr His Asn Tyr
145 150 155 160

Ala Ile Asp Trp Gln Thr Gly Ala Gly Cys Asp Ala Ala Gly Phe Phe
165 170 175

Thr Val Phe Ala Ser Glu Leu Ser Val Tyr Thr Leu Thr Ala Ile Thr
180 185 190

Leu Glu Arg Trp His Thr Ile Thr His Ala Met Gln Leu Asp Cys Lys
195 200 205

Val Gln Leu Arg His Ala Ala Ser Val Met Val Met Gly Trp Ile Phe
210 215 220

Ala Phe Ala Ala Ala Leu Phe Pro Ile Phe Gly Ile Ser Ser Tyr Met
225 230 235 240

Lys Val Ser Ile Cys Leu Pro Met Asp Ile Asp Ser Pro Leu Ser Gln
245 250 255

Leu Tyr Val Met Ser Leu Leu Val Leu Asn Val Leu Ala Phe Val Val
260 265 270

Ile Cys Gly Cys Tyr Ile His Ile Tyr Leu Thr Val Arg Asn Pro Asn
275 280 285

Ile Val Ser Ser Ser Ser Asp Thr Arg Ile Ala Lys Arg Met Ala Met
290 295 300

Leu Ile Phe Thr Asp Phe Leu Cys Met Ala Pro Ile Ser Phe Phe Ala
305 310 315 320

Ile Ser Ala Ser Leu Lys Val Pro Leu Ile Thr Val Ser Lys Ala Lys
325 330 335

Ile Leu Leu Val Leu Phe His Pro Ile Asn Ser Cys Ala Asn Pro Phe
340 345 350

Leu Tyr Ala Ile Phe Thr Lys Asn Phe Arg Arg Asp Phe Phe Ile Leu
355 360 365

Leu Ser Lys Cys Gly Cys Tyr Glu Met Gln Ala Gln Ile Tyr Arg Thr
370 375 380

Glu Thr Ser Ser Thr Val His Asn Thr His Pro Arg Asn Gly His Cys
385 390 395 400

Ser Ser Ala Pro Arg Val Thr Asn Gly Ser Thr Tyr Ile Leu Val Pro
405 410 415

Leu Ser His Leu Ala Gln Asn
420

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GTGGTGAGCA ACCCCAATGA TAAATATGAA CCCTT 35

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

GCTCACCACG CC 12

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

```
Gly Val Val Ser Xaa Xaa Xaa Xaa Xaa Asn Pro Asn Asp Lys Tyr Glu
1               5                   10                  15

Pro Phe
```

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

```
Thr Leu Asp Pro Arg Xaa Xaa Xaa Xaa Xaa Asn Pro Asn Asp Lys Tyr
1               5                   10                  15

Glu Pro Phe
```

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

```
Ser Phe Leu Leu Arg Asn
1               5
```

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 429 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

```
Met Asp Ser Lys Gly Ser Ser Gln Lys Gly Ser Arg Leu Leu Leu Leu
1               5                   10                  15

Leu Val Val Ser Asn Leu Leu Leu Cys Gln Gly Val Val Ser Asp Tyr
            20                  25                  30

Lys Asp Asp Asp Asp Lys Leu Asp Ala Thr Leu Asp Pro Arg Xaa Xaa
        35                  40                  45

Xaa Xaa Xaa Asn Pro Asn Asp Lys Tyr Glu Pro Phe Trp Glu Asp Glu
    50                  55                  60

Glu Lys Asn Glu Ser Gly Leu Thr Glu Tyr Arg Leu Val Ser Ile Asn
65                  70                  75                  80

Lys Ser Ser Pro Leu Gln Lys Gln Leu Pro Ala Phe Ile Ser Glu Asp
                85                  90                  95
```

Ala Ser Gly Tyr Leu Val Leu Tyr Tyr Leu Ala Ile Val Gly His Ser
100 105 110

Leu Ser Ile Phe Thr Leu Val Ile Ser Leu Gly Ile Phe Val Phe Phe
115 120 125

Arg Ser Leu Gly Cys Gln Arg Val Thr Leu His Lys Asn Met Phe Leu
130 135 140

Thr Tyr Ile Leu Asn Ser Met Ile Ile Ile Ile His Leu Val Glu Val
145 150 155 160

Val Pro Asn Gly Glu Leu Val Arg Arg Asp Pro Val Ser Cys Lys Ile
165 170 175

Leu His Phe Phe His Gln Tyr Met Met Ala Cys Asn Tyr Phe Trp Met
180 185 190

Leu Cys Glu Gly Ile Tyr Leu His Thr Leu Ile Val Val Ala Val Phe
195 200 205

Thr Glu Lys Gln Arg Leu Arg Trp Tyr Tyr Leu Leu Gly Trp Gly Phe
210 215 220

Pro Leu Val Pro Thr Thr Ile His Ala Ile Thr Arg Ala Val Tyr Phe
225 230 235 240

Asn Asp Asn Cys Trp Leu Ser Val Glu Thr His Leu Leu Tyr Ile Ile
245 250 255

His Gly Pro Val Met Ala Ala Leu Val Val Asn Phe Phe Phe Leu Leu
260 265 270

Asn Ile Val Arg Val Leu Val Thr Lys Met Arg Glu Thr His Glu Ala
275 280 285

Glu Ser His Met Tyr Leu Lys Ala Val Lys Ala Thr Met Ile Leu Val
290 295 300

Pro Leu Leu Gly Ile Gln Phe Val Val Phe Pro Trp Arg Pro Ser Asn
305 310 315 320

Lys Met Leu Gly Lys Ile Tyr Asp Tyr Val Met His Ser Leu Ile His
325 330 335

Phe Gln Gly Phe Phe Val Ala Thr Ile Tyr Cys Phe Cys Asn Asn Glu
340 345 350

Val Gln Thr Thr Val Lys Arg Gln Trp Ala Gln Phe Lys Ile Gln Trp
355 360 365

Asn Gln Arg Trp Gly Arg Arg Pro Ser Asn Arg Ser Ala Arg Ala Ala
370 375 380

Ala Ala Ala Ala Glu Ala Gly Asp Ile Pro Ile Tyr Ile Cys His Gln
385 390 395 400

Glu Leu Arg Asn Glu Pro Ala Asn Asn Gln Gly Glu Glu Ser Ala Glu
405 410 415

Ile Ile Pro Leu Asn Ile Ile Glu Gln Glu Ser Ser Ala
420 425

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WHAT IS CLAIMED IS:

1. A method of identifying peptide agonists or negative antagonists of a G protein coupled receptor of interest, said method comprising:

expressing a peptide of a peptide library tethered to a G protein coupled receptor of interest in a cell; and

monitoring said cell to determine whether the peptide is an agonist or negative antagonist of said G protein coupled receptor of interest.

2. The method of claim 1 for identifying a peptide agonist wherein expressing a peptide of a peptide library tethered to a G protein coupled receptor of interest in a cell comprises:

preparing a G protein coupled receptor construct for identifying a peptide agonist, the G protein coupled receptor construct comprising:

a nucleic acid molecule encoding a G protein coupled receptor with a deleted first amino terminus;

a nucleic acid molecule encoding a second amino terminus of a self-activating receptor attached to said nucleic acid molecule encoding a G protein coupled receptor at the deleted first amino terminus, said second amino terminus having a deleted portion which is a peptide agonist for activating said self-activating receptor; and

a nucleic acid molecule encoding the peptide of the peptide library inserted into said second amino terminus and replacing said deleted portion;

introducing the G protein coupled receptor construct into a cell;

allowing said cell to express said G protein coupled receptor encoded thereby; and

exposing said cell to a ligand of said self-activating receptor, wherein said ligand cleaves said G protein coupled receptor construct to expose said inserted peptide of said peptide library.

3. The method of claim 2 wherein said introducing comprises injecting said G protein coupled receptor construct into said cell.

4. The method of claim 2 wherein said introducing comprises transformation of said cell with an expression vector, said expression vector comprising said G protein coupled receptor construct.

5. The method of claim 1 for identifying a peptide negative antagonist wherein said G protein coupled receptor of interest is a constitutively active G protein coupled receptor and wherein expressing a peptide of a peptide library tethered to the G protein coupled receptor of interest in a cell comprises:

preparing a constitutively active G protein coupled receptor construct for identifying a peptide negative antagonist, the constitutively active G protein coupled receptor construct comprising:

a nucleic acid molecule encoding a constitutively active G protein coupled receptor with a deleted first amino terminus;

a nucleic acid molecule encoding a second amino terminus of a self-activating receptor attached to said nucleic acid molecule encoding said constitutively active G protein coupled receptor at the deleted first amino terminus, said second amino terminus having a deleted portion which includes a peptide agonist for activating said self-

activating receptor as well as any amino acids positioned amino terminally to the peptide agonist; and

a nucleic acid molecule encoding the peptide of the peptide library inserted into said second amino terminus and replacing said deleted portion;

introducing the constitutively active G protein coupled receptor construct into a cell; and

allowing said cell to express said constitutively active G protein coupled receptor encoded thereby.

6. The method of claim 5 wherein said introducing comprises injecting said constitutively active G protein coupled receptor construct into said cell.

7. The method of claim 5 wherein said introducing comprises transformation of said cell with an expression vector, said expression vector comprising said constitutively active G protein coupled receptor construct.

8. The method of claim 1 for identifying a peptide agonist wherein expressing a peptide of a peptide library tethered to the G protein coupled receptor of interest in a cell comprises:

preparing a G protein coupled receptor construct for identifying a peptide agonist, the G protein coupled receptor construct comprising:

a nucleic acid molecule encoding a G protein coupled receptor with a deleted first amino terminus;

a nucleic acid molecule encoding a second amino terminus of a self-activating receptor attached to said nucleic acid

molecule encoding said G protein coupled receptor at the deleted first amino terminus, said second amino terminus having a deleted portion which includes a peptide agonist for activating said self-activating receptor as well as any amino acids positioned amino terminally to the peptide agonist; and

a nucleic acid molecule encoding the peptide of the peptide library inserted into said second amino terminus and replacing said deleted portion;

introducing the G protein coupled receptor construct into a cell; and

allowing said cell to express said G protein coupled receptor encoded thereby.

9. The method of claim 8 wherein said introducing comprises injecting said G protein coupled receptor construct into said cell.

10. The method of claim 8 wherein said introducing comprises transformation of said cell with an expression vector, said expression vector comprising said G protein coupled receptor construct.

11. The method of claim 1 wherein said G protein coupled receptor signals through an ion channel pathway and wherein said monitoring comprises detecting levels of said ion within said cell.

12. The method of claim 11 wherein said ion channel pathway is a calcium channel.

13. The method of claim 12 wherein said cell is a *Xenopus* oocyte and wherein said monitoring comprises voltage clamp analysis.

14. The method of claim 1 wherein said G protein coupled receptor signals through a cyclic adenosine monophosphate pathway and wherein said monitoring comprises detecting levels of cyclic adenosine monophosphate within said cell.

15. A G protein coupled receptor construct for identifying a peptide agonist of the G protein coupled receptor, the construct comprising:

a nucleic acid molecule encoding a G protein coupled receptor with a deleted first amino terminus;

a nucleic acid molecule encoding a second amino terminus of a self-activating receptor attached to said nucleic acid molecule encoding a G protein coupled receptor at the deleted first amino terminus, said second amino terminus having a deleted portion which is a peptide agonist for activating said self-activating receptor; and

a nucleic acid molecule encoding a peptide of a peptide library inserted into said second amino terminus and replacing said deleted portion.

16. The G protein coupled receptor construct of claim 15 wherein said self-activating receptor is a thrombin receptor.

17. The G protein coupled receptor construct of claim 16 wherein said second amino terminus of a nucleic acid molecule encoding a thrombin receptor encodes an amino acid sequence as shown in SEQ ID NO:1, and wherein amino acid residues 9 to 13 of SEQ ID NO:1 are the portion which is a peptide agonist for said thrombin receptor.

18. The G protein coupled receptor construct of claim 15 wherein the G protein coupled receptor is a human calcitonin receptor.

19. The G protein coupled receptor construct of claim 18 wherein said construct has an amino acid sequence as shown in SEQ ID NO:44, and wherein amino acid residues 47 to 51 of SEQ ID NO:44 are the peptide of a peptide library, amino acid residues 1 to 101 of SEQ ID NO:44 are the second amino terminus, and amino acid residues 102 to 429 of SEQ ID NO:44 are the nucleic acid molecule encoding the human calcitonin receptor with said first amino terminus deleted.

20. The G protein coupled receptor construct of claim 15 wherein the G protein coupled receptor is a human follicle stimulating hormone receptor.

21. The G protein coupled receptor construct of claim 20 wherein said construct has an amino acid sequence as shown in SEQ ID NO:2, and wherein amino acid residues 47 to 51 of SEQ ID NO:2 are the peptide of a peptide library, amino acid residues 39 to 101 of SEQ ID NO:2 are the second amino terminus, and amino acid residues 102 to 436 of SEQ ID NO:2 are the nucleic acid molecule encoding the human follicle stimulating hormone receptor with said first amino terminus deleted.

22. A cell comprising the G protein coupled receptor construct of claim 15.

23. The cell of claim 22 wherein the cell is a *Xenopus* oocyte.

24. An expression vector comprising the G protein coupled receptor construct of claim 15.

25. The expression vector of claim 24 wherein said expression vector is selected from the group consisting of a plasmid and a virus.

26. A cell comprising the expression vector of claim 24.

27. A constitutively active G protein coupled receptor construct for identifying a peptide negative antagonist of the constitutively active G protein coupled receptor, the construct comprising:

a nucleic acid molecule encoding a constitutively active G protein coupled receptor with a deleted first amino terminus;

a nucleic acid molecule encoding a second amino terminus of a self-activating receptor attached to said nucleic acid molecule encoding a constitutively active G protein coupled receptor at the deleted first amino terminus, said second amino terminus having a deleted portion which includes a peptide agonist for activating said self-activating receptor as well as any amino acids positioned amino terminally to the peptide agonist; and

a nucleic acid molecule encoding a peptide of a peptide library inserted into said second amino terminus and replacing said deleted portion.

28. The constitutively active G protein receptor construct of claim 27 wherein said self-activating receptor is a thrombin receptor.

29. The constitutively active G protein coupled receptor construct of claim 28 wherein said second amino terminus of a nucleic acid molecule encoding a thrombin receptor encodes an amino acid sequence as shown in SEQ ID NO:1, and wherein amino acid residues 9 to 13 of SEQ ID NO:1 are the portion which is a peptide agonist for said thrombin receptor.

30. A cell comprising the constitutively active G protein coupled receptor construct of claim 27.

31. The cell of claim 30 wherein the cell is a *Xenopus* oocyte.

32. The cell of claim 30 wherein the cell is a yeast cell.

33. An expression vector comprising the constitutively active G protein coupled receptor construct of claim 27.

34. The expression vector of claim 33 wherein said expression vector is selected from the group consisting of a plasmid and a virus.

35. A cell comprising the expression vector of claim 34.

36. A G protein coupled receptor construct for identifying a peptide agonist of the G protein coupled receptor, the construct comprising:

a nucleic acid molecule encoding a G protein coupled receptor with a deleted first amino terminus;

a nucleic acid molecule encoding a second amino terminus of a self-activating receptor attached to said nucleic acid molecule encoding a G protein coupled receptor at the deleted first amino terminus, said second amino terminus having a deleted portion which includes a peptide agonist for activating said self-activating receptor as well as any amino acids positioned amino terminally to the peptide agonist; and

a nucleic acid molecule encoding a peptide of a peptide library inserted into said second amino terminus and replacing said deleted portion.

37. The G protein receptor construct of claim 36 wherein said self-activating receptor is a thrombin receptor.

38. The G protein coupled receptor construct of claim 37 wherein said second amino terminus of a nucleic acid molecule encoding a thrombin receptor encodes an amino acid sequence as shown in SEQ ID NO:1, and wherein amino acid residues 9 to 13 of SEQ ID NO:1 are the portion which is a peptide agonist for said thrombin receptor.

39. The G protein coupled receptor construct of claim 36 wherein the G protein coupled receptor is a human calcitonin receptor.

40. The G protein coupled receptor construct of claim 39 wherein said construct has an amino acid sequence as shown in SEQ ID NO:44, and wherein amino acid residues 47 to 51 of SEQ ID NO:44 are the peptide of a peptide library, amino acid residues 1 to 101 of SEQ ID NO:44 are the second amino terminus, and amino acid residues 102 to 429 of SEQ ID NO:44 are the nucleic acid molecule encoding the human calcitonin receptor with said first amino terminus deleted.

41. The G protein coupled receptor construct of claim 36 wherein the G protein coupled receptor is a human follicle stimulating hormone receptor.

42. The G protein coupled receptor construct of claim 41 wherein said construct has an amino acid sequence as shown in SEQ ID NO:2, and wherein amino acid residues 47 to 51 of SEQ ID NO:2 are the peptide of a peptide library, amino acid residues 39 to 101 of SEQ ID NO:2 are the second amino terminus, and amino acid residues 102 to 436 of SEQ ID NO:2 are the nucleic

acid molecule encoding the human follicle stimulating hormone receptor with said first amino terminus deleted.

43. A cell comprising the G protein coupled receptor construct of claim 36.

44. The cell of claim 43 wherein the cell is a yeast cell.

45. An expression vector comprising the G protein coupled receptor construct of claim 36.

46. The expression vector of claim 45 wherein said expression vector is selected from the group consisting of a plasmid and a virus.

47. A cell comprising the expression vector of claim 45.

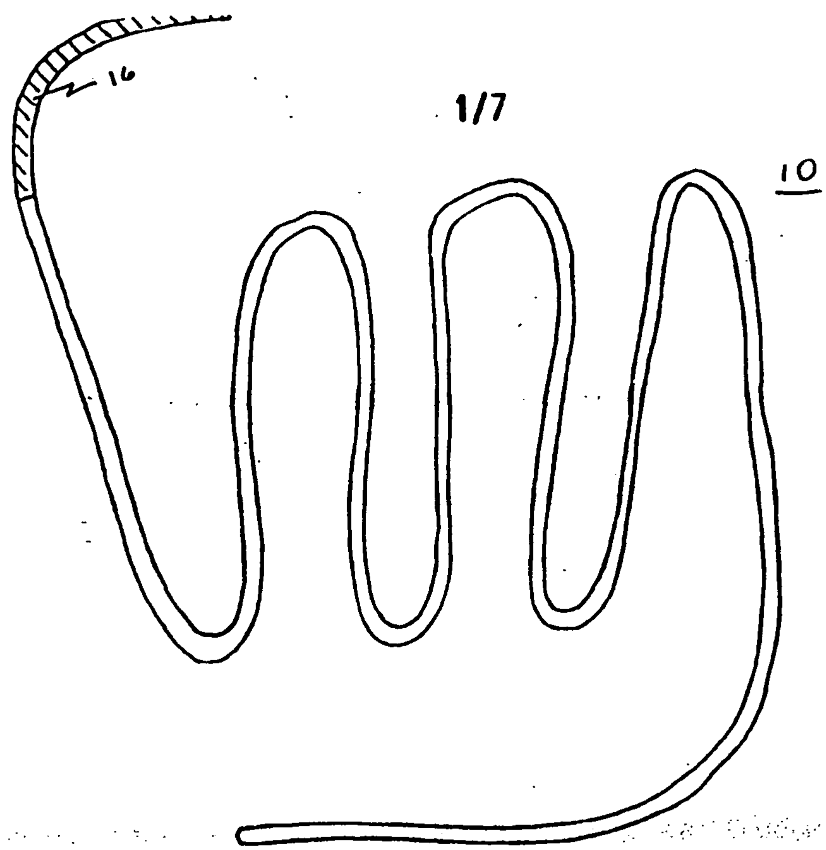


Fig. 1

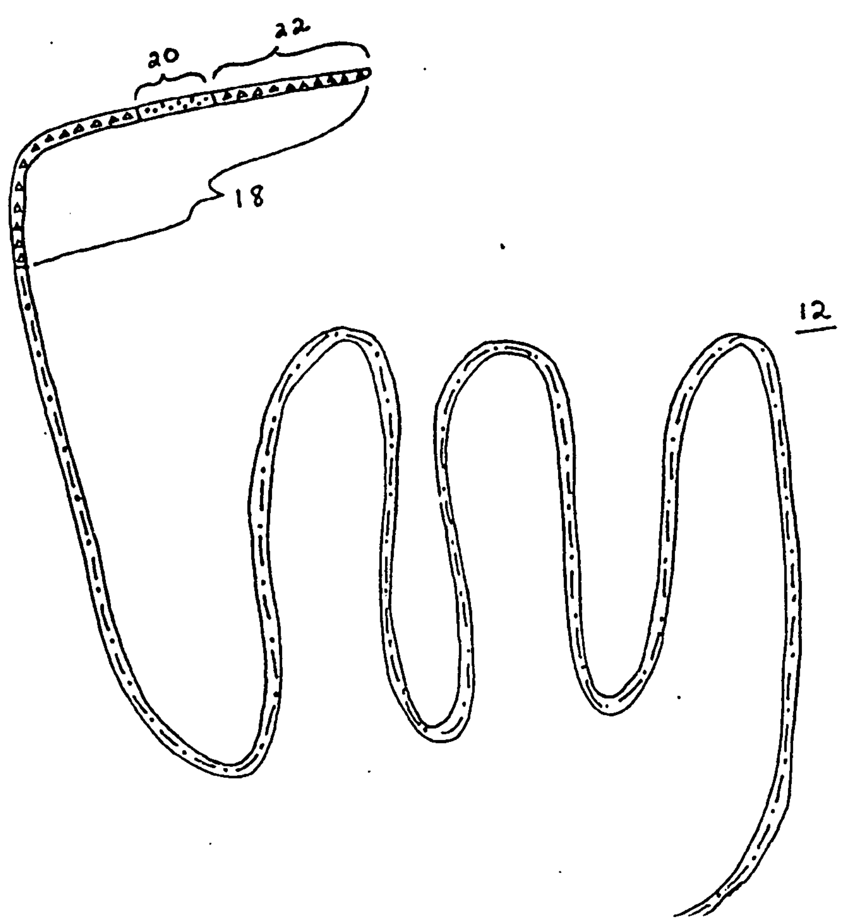


Fig. 2

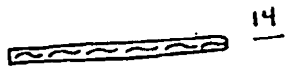


Fig. 3

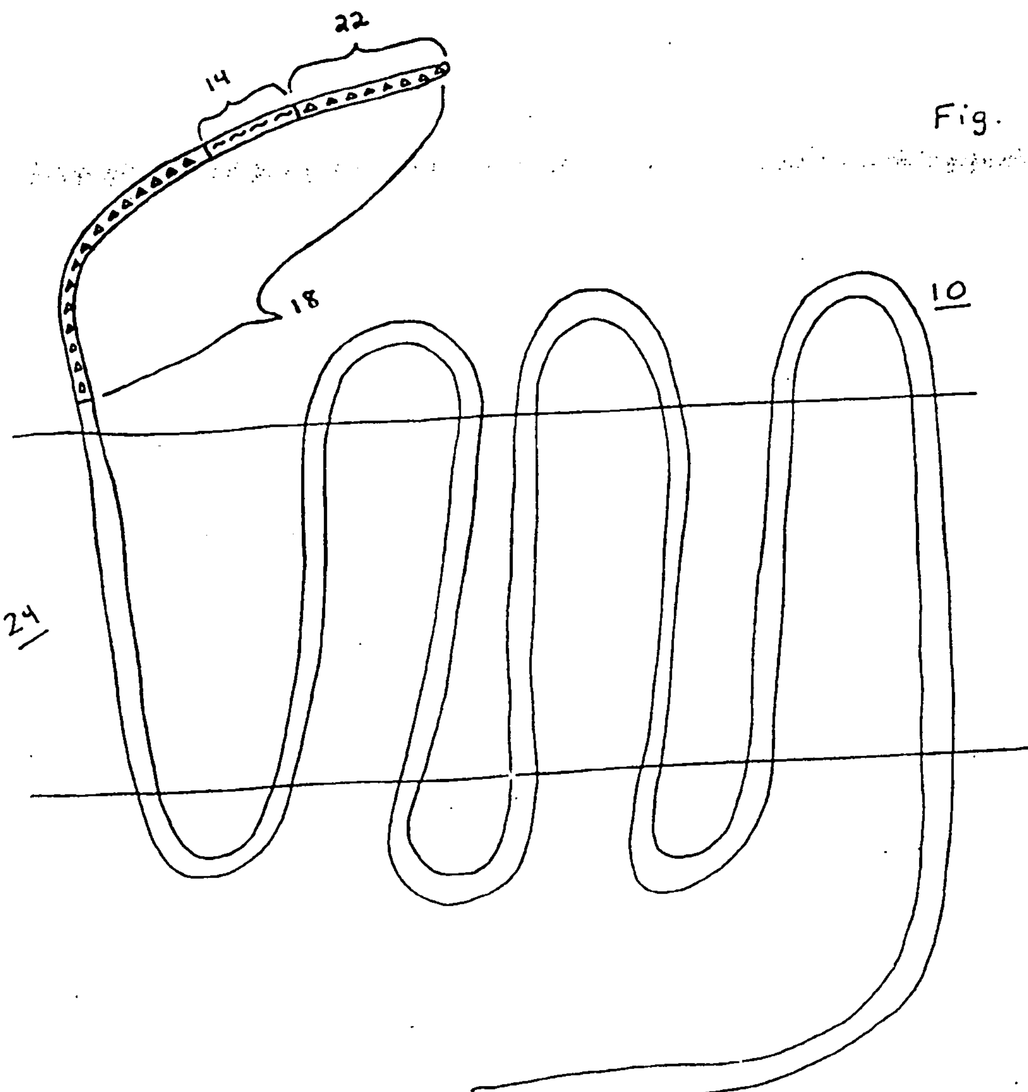


Fig. 4

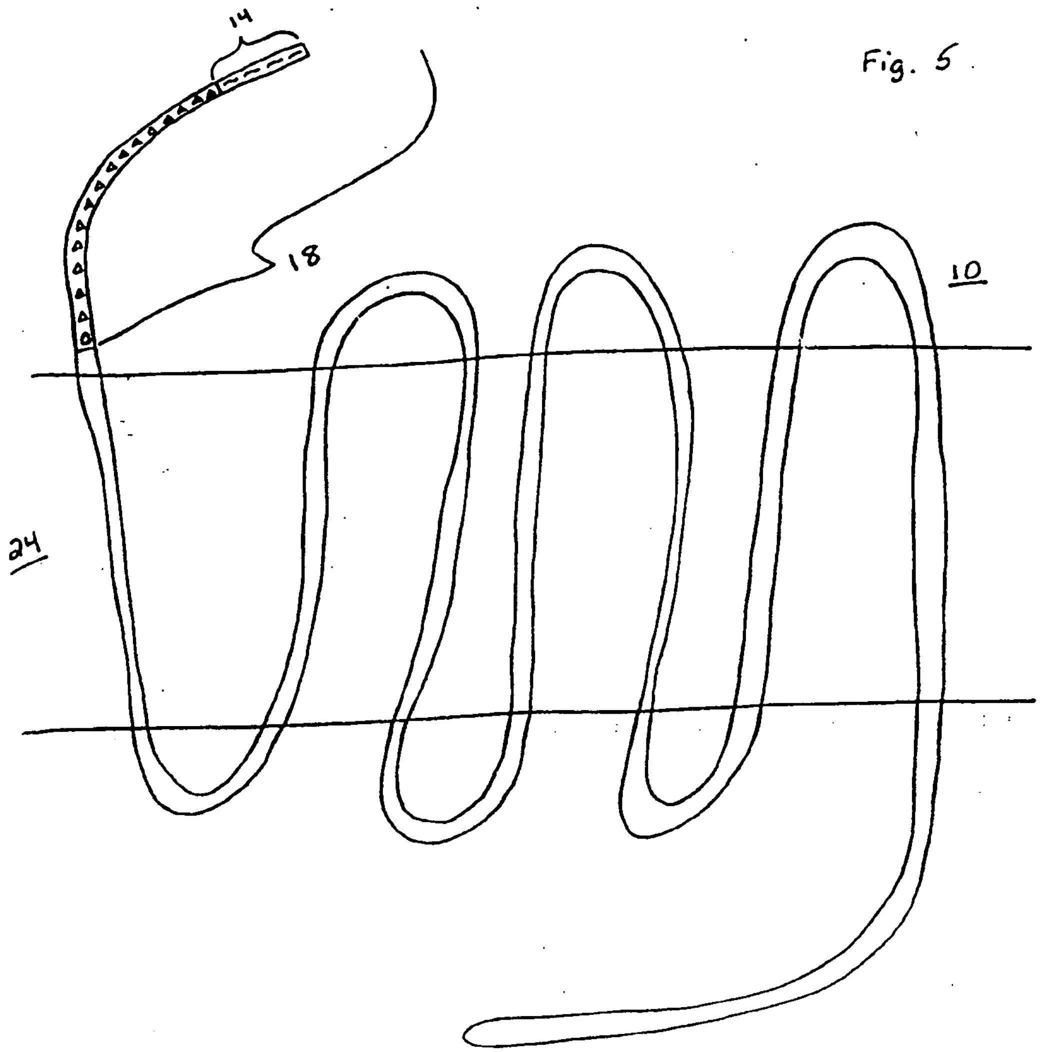


Fig. 5

Fig. 6

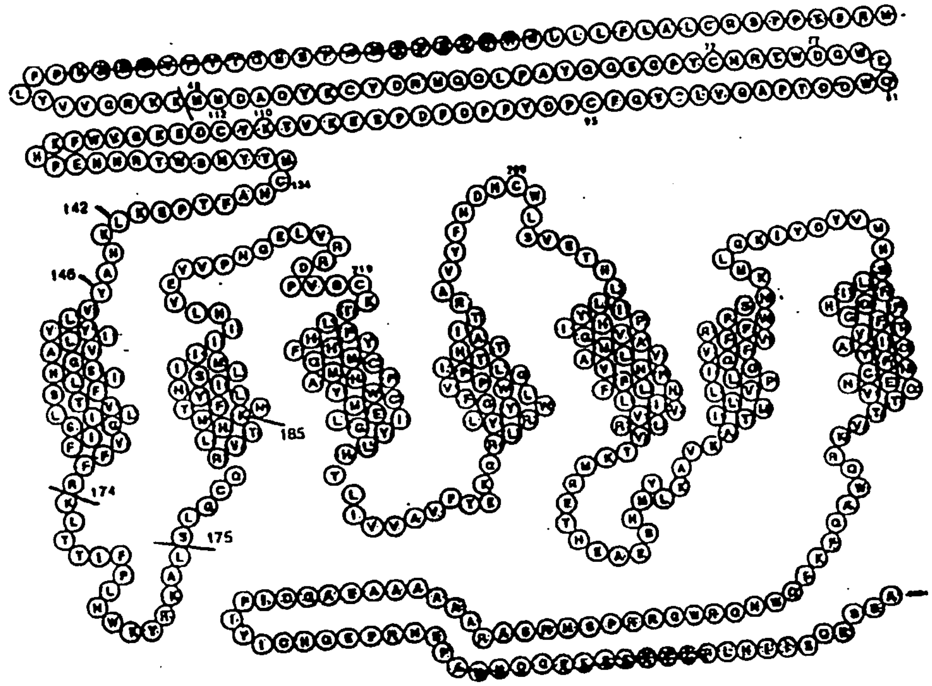
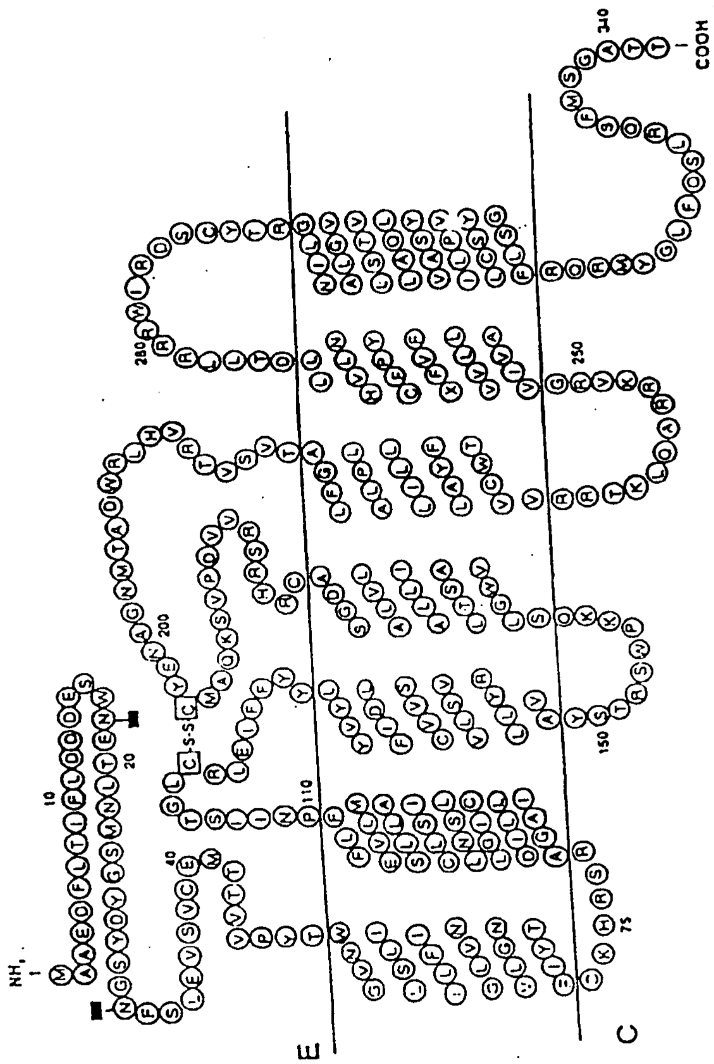
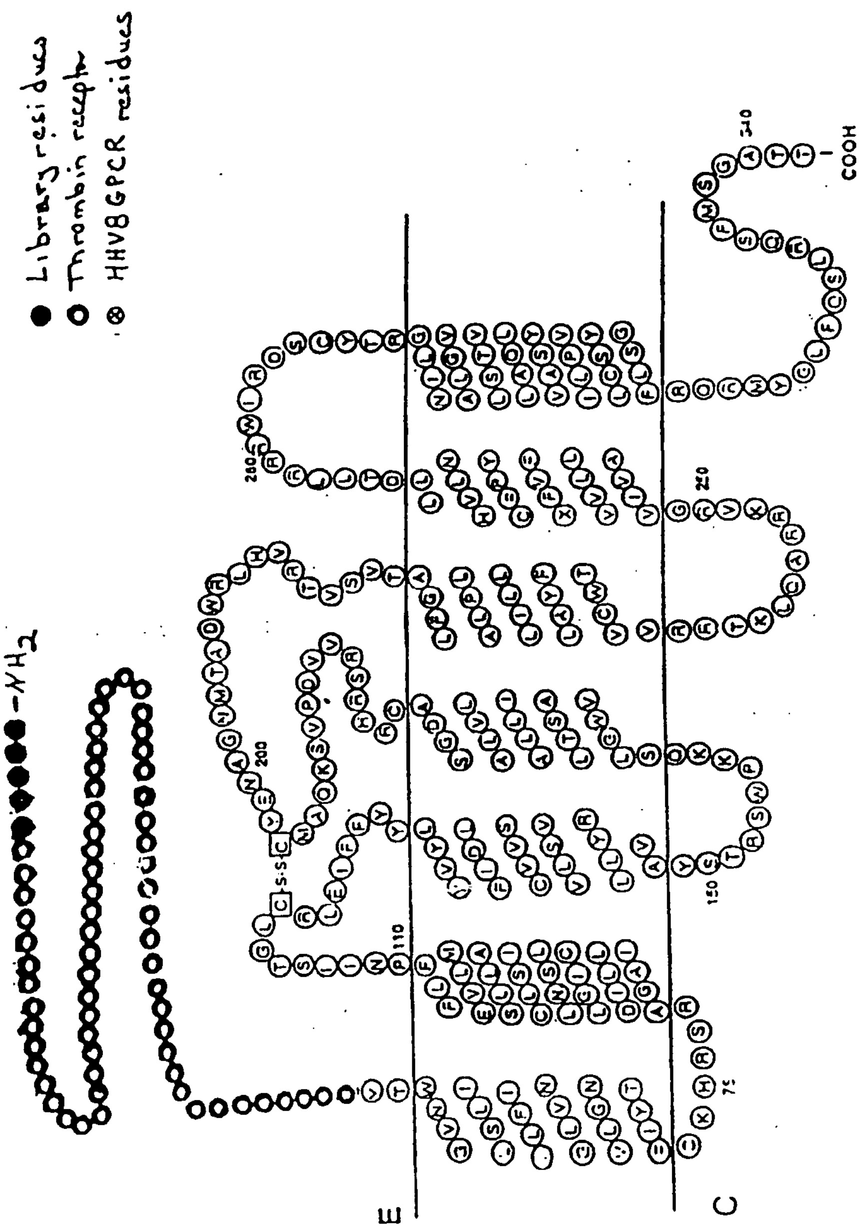


Fig. 7

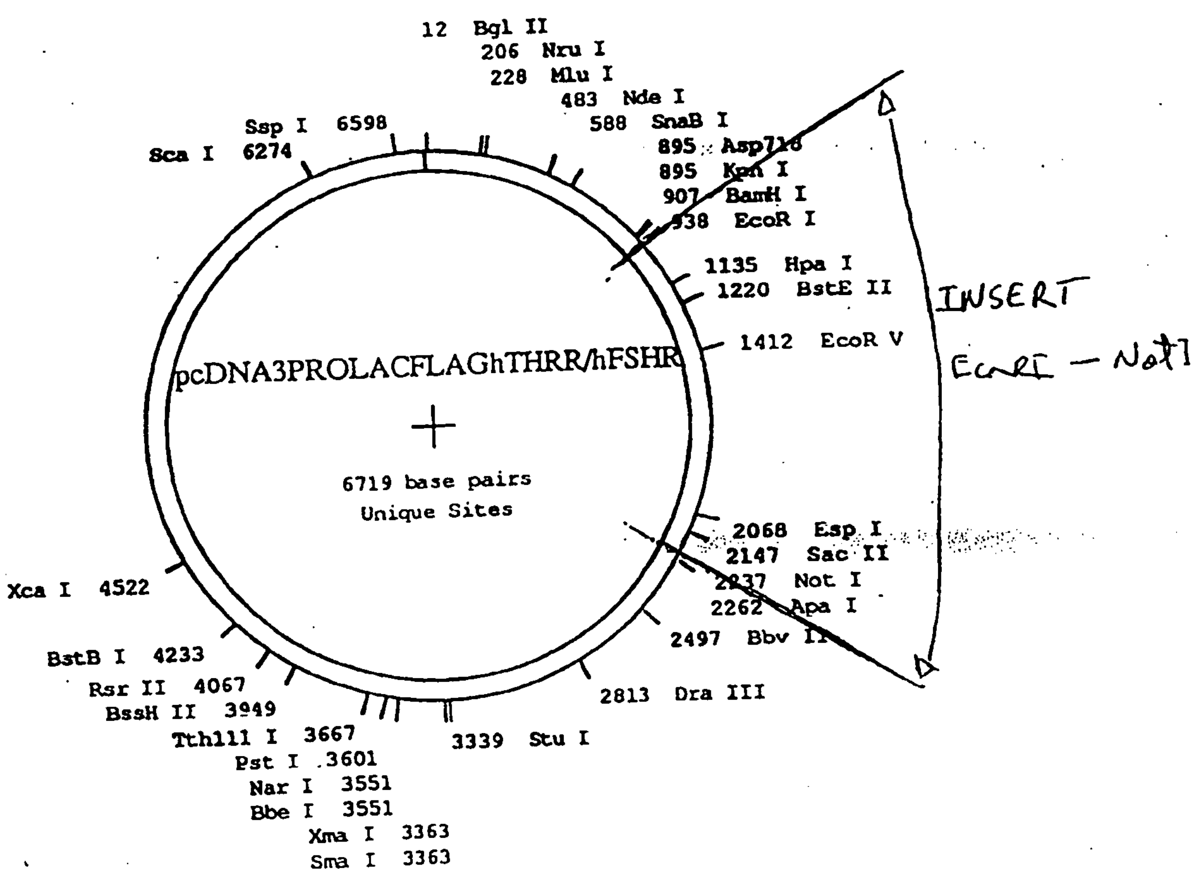




● Library residues
○ Thrombin receptor
⊗ HHV8 GPCR residues
-NH2
COOH
E
C

Fig. 8

Fig. 9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206 Nru I
228 Mlu I
483 Nde I
588 SnaB I
895 Asp718
895 Kpn I
907 BamH I
938 EcoR I
1135 Hpa I
1220 BstE II
1412 EcoR V
2068 Esp I
2147 Sac II
2237 Not I
2262 Apa I
2497 Bbv II
2813 Dra III
3339 Stu I
Sma I 3363
Xma I 3363
Bbe I 3551
Nar I 3551
Pst I 3601
Tth111 I 3667
BssH II 3949
Rsr II 4067
BstB I 4233
Xca I 4522
Sca I 6274
Ssp I 6598
pcDNA3PROLACFLAGhTHRR/hFSHR
6719 base pairs
Unique Sites
INSERT
EcoRI – NotI

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/02377

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.2, 69.1, 320, 325, 252.3, 254.11; 514/2; 530/300, 350; 536/23.1, 23.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to claim No.
A	US 5,482,835 A (KING ET AL) 09 January 1996, entire document.	1-12, 14-22, 14-30, 32-47
A	PRICE ET AL. Functional coupling of a mammalian somatostatin receptor to the yeast pheromone response pathway. Mol. Cell. Biol. November 1995. Vol. 15, No. 11, pages 6188-6195, especially abstract, Figure 1, and page 6189.	1-12, 14-22, 24-30, 32-47
A	JULIUS ET AL. Molecular characterization of a functional cDNA encoding the serotonin 1c receptor. Science. 29 July 1988. Vol. 241, pages 558-564, especially Figure 1.	1-31, 33-43, 45-47

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
02 APRIL 1998	29 MAY 1998

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/02377

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WRIGHT ET AL. Cloning strategies for peptide hormone receptors. Acta. Endocrinol. 10 March 1992. Vol. 125, pages 97-104, entire document.	1-47
A	CHEN ET AL. Tethered ligand library for discovery of peptide agonists. J. Biol. Chem. 06 October 1995. Vol. 270, No. 40, pages 23398-23401, especially Figure 1.	1-47